Effect of mutations of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase at tryptophan-110 on enantioselectivity of reduction of phenyl –ring-containing ketones

BY

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This thesis, written by **Odey Falah Bsharat** under the direction of his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN CHEMISTRY**.

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Dedicated to my beloved parents whose continuous prayers and inspiration led to the accomplishment of this study

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my father Falah Bsharat, and my mother Nimah Bsharat who raised me and gave me the strength and wisdom to accomplish this yet another goal in my life.

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LIST OF ABBREVIATIONS

ADH	:	Alcohol dehydrogenase
DDT	:	Dithiothreitol
ee	:	Enantiomeric excess
EI MS	:	Electron Ionization Mass Spectrometry
GC	:	Gas Chromatography
HPLC	:	High Performance Liquid Chromatography
HLADH	:	Horse liver alcohol dehydrogenase
K_m	:	Michaelis–Menten constant
LkADH	:	*Lactobacillus kefir* alcohol dehydrogenase
NMR	:	Nuclear Magnetic Resonance
NADH	:	Nicotinamide-adenine dinucleotide
NADPH	:	Nicotinamide-adenine dinucleotide phosphate
TeSADH	:	*Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase
TbADH	:	*Thermoanaerobium brockii* secondary alcohol dehydrogenase
TbADH	:	*Thermoanaerobium brockii* secondary alcohol dehydrogenase
Tris-HCl	:	Tris(hydroxymethyl)aminomethane hydrochloride
T_4HNR	:	tetrahydroxy naphthalene reductase
T_3HNR	:	trihydroxy naphthalene reductase
V_{max}	:	Maximum velocity
YADH	:	Yeast alcohol dehydrogenase

ABSTRACT

Full Name : Odey Falah Bsharat

Thesis Title : Effect of mutations of Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase at tryptophan-110 on enantioselectivity of reduction of phenyl–ring-containing ketones

Major Field : Chemistry

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The asymmetric reduction of selected phenyl-ring-containing ketones by various mutants of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (TeSADH) was studied using single and dual site mutagenesis. The expansion of both small and large pockets of TeSADH in the mutant W110A/I86A not only accommodates the substrates of single mutants W110A and I86A within the active site, but also expands the substrate scope to ketones bearing two sterically demanding groups (bulky-bulky ketones); which are not substrates for TeSADH single mutants. We also explored the regio- and enantioselective reduction of diketones using W110A/I86A TeSADH. The double mutant exhibited dual stereopreferences that resulted in Prelog products and the *anti*-Prelog products were occasionally observed. Kinetic parameters, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), for the reduction reactions of substituted 4-phenyl-2-butanone and 1-phenyl-2-propanone were consistent with that of the enantioselectivity values. Moreover, we conducted asymmetric reduction of substituted 2-tetralones, using various mutants of TeSADH which revealed that the stereoselectivity and its magnitude were dependent on the position of substituent on the aromatic ring; such an outcome has been attributed due to different binding modes of these substrates in the enzyme active site. In addition, changing the position of the substituent on the aromatic ring also has a

great impact on the binding affinity and maximum catalytic rate, as reflected by the kinetic parameters V_{max} and K_m.

ملخص الرسالة

الاسم الكامل:عدي فلاح بشارات

عنوان الرسالة: تاثير طفرات مختلفة من الانزيم (TeSADH) على موقع Trp110 على الاختزال غير المتماثل لمركبات الكيتون التي تحتوي على حلقة اروماتية

التخصص: كيمياء

تاريخ الدرجة العلمية:2016

تم تحضير مركبات الكحول من الاختزال غير المتماثل لمجموعة من مركبات الكيتون التي تحتوي على حلقة اروماتية باستخدام طفرات مختلفة من الانزيم (TeSADH) . باستخدام الطفرة الثنائية للموقع النشط للانزيم تمكنا من اختزال جميع مركبات كيتون التي يمكن اختزالها بالطفرة الاحادية وكذلك تمكن من زيادة عدد مركبات الكيتون التي يمكن ان تختزل بواسطة الانزيم وخصوصا مركبات الكيتون التي تحتوي على مجموعات اروماتية حول الكاربونيل. كذلك تمكنا من اختزال مركبات كيتون التي تحتوي على مجموعتين كاربونيل بفعالية عالية. تم قياس المعاملات الحركية (V_{max} and K_m) لمركبات ٤- فينيل-٢ - بيوتانون و١- فينيل- ٢- بروبانون وكانت متوافقة مع قيم (*ee*) التي تم ايجادها. بالاضافة الى ذلك تم اجراء اختزال غير متماثل لمركبات (2-tetralones) ومشتقاتها ووجدنا ان هناك تاثير كبير لموقع مجموعة التفرع على الحلقة الاروماتية على نوع الكحول الناتج وتم قياس معاملات الحركية (V_{max} and K_m) لهذه المركبات.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Optically active alcohols are very important synthons in the pharmaceutical, agrochemical, food, and petroleum industries. They are generally produced in industry from their racemates via kinetic resolution (KR), where two enantiomers react with different reaction rates in a chemical reaction, resulting in an enantioenriched alcohol. However, this approach has a drawback of being limited to a maximum yield of 50% pure enantiomeric product [1, 2]. Deracemization of racemic alcohols is an attractive approach for the production of optically active alcohols in 100% theoretical yield [3, 4], and thus considered as a potential alternative to KR. However, all reported deracemization approaches require two compatible reaction systems to work in one pot, which makes searching for new deracemization methods a challenge. Another approach for producing optically active alcohols is asymmetric reduction of their corresponding ketones. This can be done chemically or using biocatalysis; however, the use of biocatalysis is preferred for several reasons. First, enzymes have high regio-, chemo-, and steroselectivity. Second, their application in fine chemical industry has been spreading very fast because it cures industrial challenges such as cost-effectiveness and sustainability .Third, enzymes are environmentally benign since they are natural catalysts

and they work under mild conditions, which reduce side effects like epimerization and isomerization [5].

Asymmetric reduction of pro-chiral ketones that contain bulky groups on both sides of the carbonyl, like 2-tetralone and its phenyl-substituted analogs, is of great interest because most of the reported methods for their asymmetric reduction are either poorly stereoselective or non-stereoselective [6]. This calls for methods that can reduce such compounds with high enantioselectivities. One important class of reactions is biocatalytic asymmetric reduction catalyzed by (ADHs) in which nicotinamide-adenine dinucleotide (NADH) or its phosphate (NADPH) act as hydride sources (Figure 1). Commercially available ADHs have been employed efficaciously in asymmetric redox reactions. For example, yeast ADH (YADH), horse liver ADH (HLADH), *Thermoanaerobium brockii* ADH (TbADH) [7] and *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH) [8] have been employed in the enantioselective reduction and enantiospecific oxidation of aromatic ketones and alcohols, respectively [9].

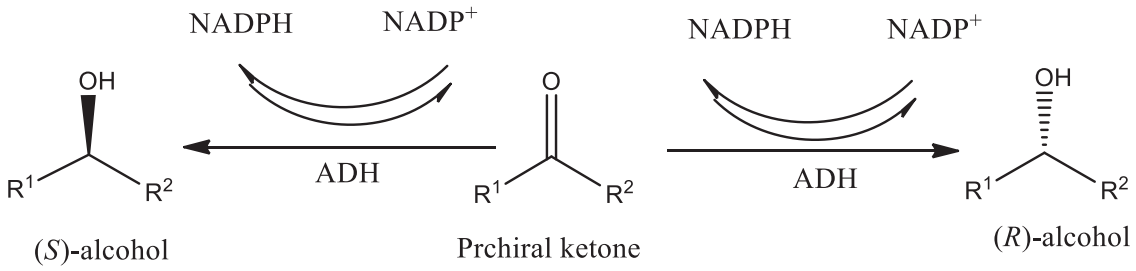
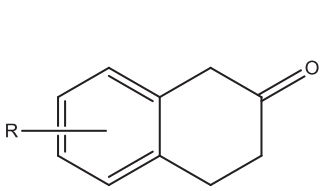
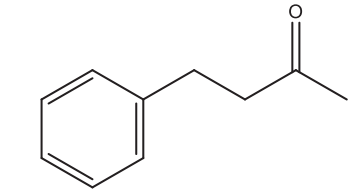


Figure 1: The asymmetric reduction of prochiral ketones catalyzed by alcohol dehydrogenases (ADHs). R^1 has higher Cahn-Ingold-Prelog priority than R^2.

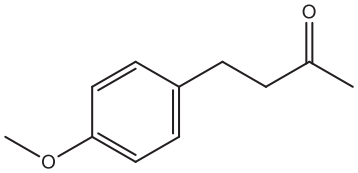
Although these reactions are enantioselective and lead to optically active alcohols with high enantiomeric excess (*ee*), the high substrate specificity of ADHs is still a major challenge that is restricting their use in organic synthesis. Herein, we propose the use of various mutants of TeSADH in the asymmetric reduction of 2-tetralone derivatives and other phenyl ring containing ketones shown in Figure 2.



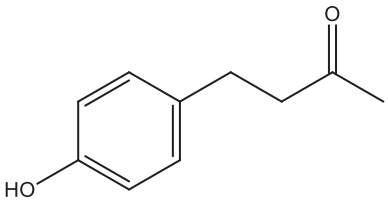
R:H ,2-tetralone (**1a**)
R:5-OMe, 5-methoxy-2-tetralone (**1b**)
R:7-OMe, 7-methoxy-2tetralone (**1c**)
R:8-OMe, 8-methoxy-2-tetralone (**1d**)
R:6-Chloro, 6-chloro-2-tetralone(**1e**)



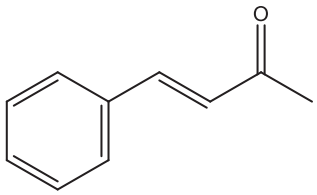
4-phenylbutan-2-one4-phenylbutan-2-one (**1f**)



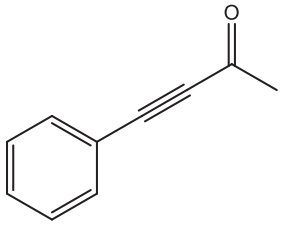
4-(4-methoxyphenyl)butan-2-one (**1g**)



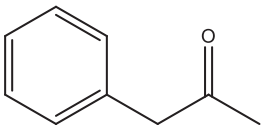
4-(4-hydroxyphenyl)butan-2-one (**1h**)



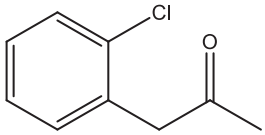
4-Phenyl-3-buten-2-one (**1i**)



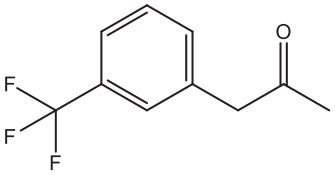
4-Phenyl-3-butyn-2-one (**1j**)



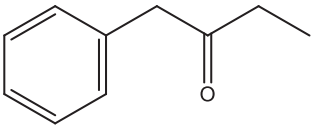
1-phenylpropan-2-one (**1k**)



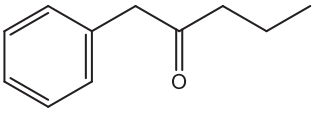
1-(2-chlorophenyl)propan-2-one (**1l**)



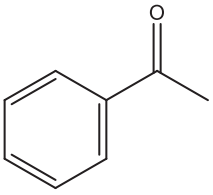
1-(3-(trifluoromethyl)phenyl)propan-2-one (**1m**)



1-phenylbutan-2-one (**1n**)



1-phenylpentan-2-one (**1o**)



acetophenone (**1p**)

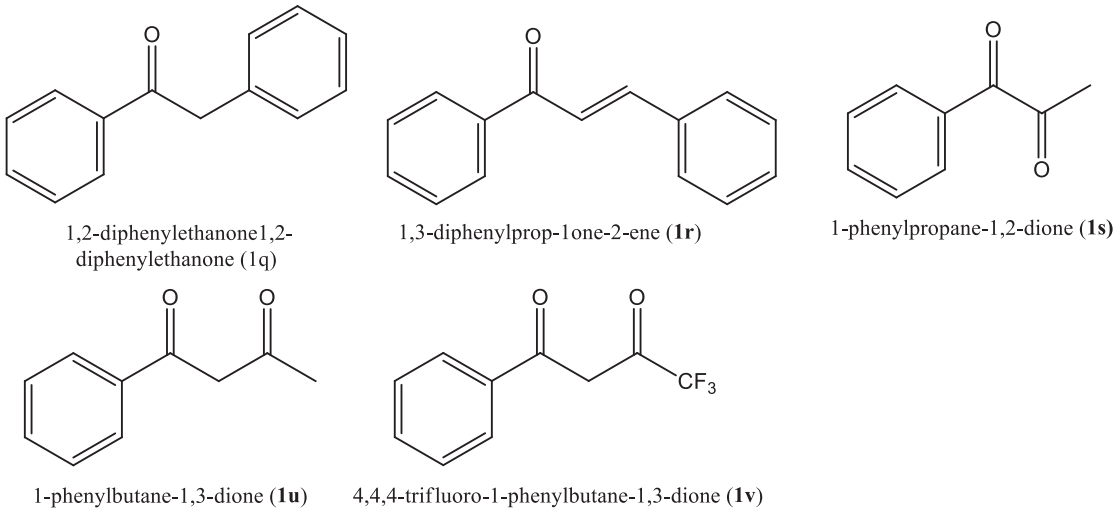


Figure 2: Different substrates studied with various mutants of TeSADH.

1.2 Alcohol Dehydrogenases

Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X = 1 or 2) catalyze the reversible asymmetric reduction of ketones and aldehydes to their corresponding alcohols. In living organisms, these reactions require co-enzymes, usually NADPH or NADH, which act as hydride sources for reduction and their corresponding oxidized forms ($NADP^+$ or NAD^+) are used for oxidation reactions. Moreover, enantiomerically pure alcohols are important building blocks for agricultural and bioactive pharmaceuticals [10].

TeSADH is an important ADH, which has been successfully employed in the reversible asymmetric reduction of ketones. This enzyme has gained significant interest because of its high thermal stability and tolerance to various organic solvents [11]. The active site of TbADH is identical to the active site of TeSADH proposed by Keinan that [12], consists of two pockets, one large and one small; the smaller pocket possesses a higher affinity for alkyl chains than the large pocket, although it cannot accommodate

sterically bulkies groups than isopropyl [13]. The crystal structure of TbADH was discovered in 1998 [14], which provided a better understanding of the active site architecture and enabled the design of useful mutants of TeSADH. An interesting site for mutations turned to be Trp-110, which forms part of the large pocket, as shown in Figure 3 [15]. Musa *et al.* has earlier reported that the W110A mutation alters the substrate specificity of TeSADH to accommodate phenyl-ring-containing alcohols and their corresponding ketones in agreement with Prelog's rule [16].On the other hand substrate specificity for ADHs depends on the nature of the aryl/alkyl group attached to alpha carbon of the secondary alcohol or their corresponding ketones. In attempt to expand its substrate specificity, several mutants of TeSADH were designed and revealed to recognize sterically-hindered groups such as phenyl rings or long alkyl chains, that are not recognize by wild-type TeSADH. Among them W110G, W110A, W110V, and W110A/I86ATeSADH, which used in this study.

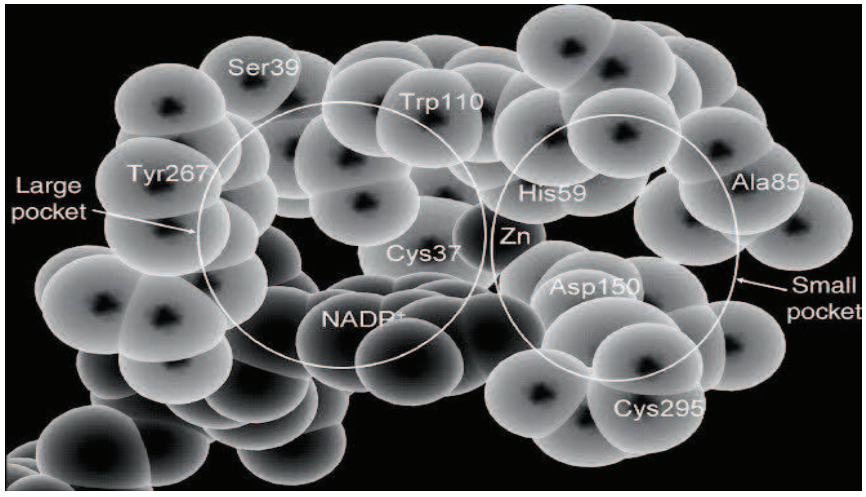


Figure 3: The structure of the active site of TeSADH and TbADH (*Protein Eng. Des.* 2007, *20*, 47-55).

1.3. ADH-Catalyzed Asymmetric redox reactions

Asymmetric reduction of ketones involves the addition of two hydrogen atoms to a carbonyl group that results in forming an optically active alcohol. In ADH-catalyzed reduction of ketones, the pro-*R* or pro-*S* hydride of the NAD(P)H is delivered either from the *Re* face or *Si* face of a prochiral ketone, leading to formation of corresponding alcohol with *S* or *R* configuration, respectively, if the larger group (R_2) has higher Cahn-Ingold-Prelog priority than the smaller group (R_1), The pro-*R* hydride NAD(P)H is delivered from the *Re* face to produce the corresponding *S* in most the cases [17]. Such a stereochemical outcome is referred to Prelog's rule. For example, yeast ADH, horse liver ADH, TbADH and TeSADH are considered as Prelog ADHs. Whereas, *Lactobacillus kefir* ADH is known as an *anti*-Prelog ADHs, a rare type of ADHs. The stereochemistry of hydride addition in ADH-catalyzed reduction of ketones is depicted in Figure 4.

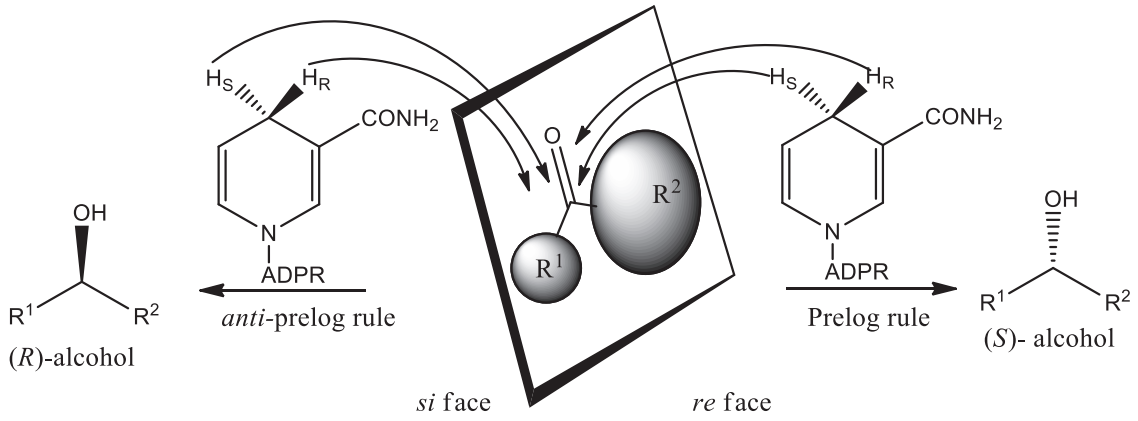


Figure 4: The stereochemistry of hydride addition in ADH-catalyzed reduction of ketones. ADPR = adenosine diphosphoribose. R^2 has higher Cahn-Ingold-Prelog priority than R^1.

TeSADH is known to be an effective biocatalyst for various synthetic applications [18] owing to the fact that it accepts alcohols and their corresponding ketones as substrates with high activities and stereoselectivities. In addition it withstands denaturation in high concentrations of organic solvents [19], which, in turn, allow it the use of cosubstrates like acetone and 2-propanol in the oxidation and reduction pathways, respectively, in a reasonable concentrations for the regeneration of coenzyme, to makes the process catalytic (Figure 5), Moreover, TeSADH is thermally stable.

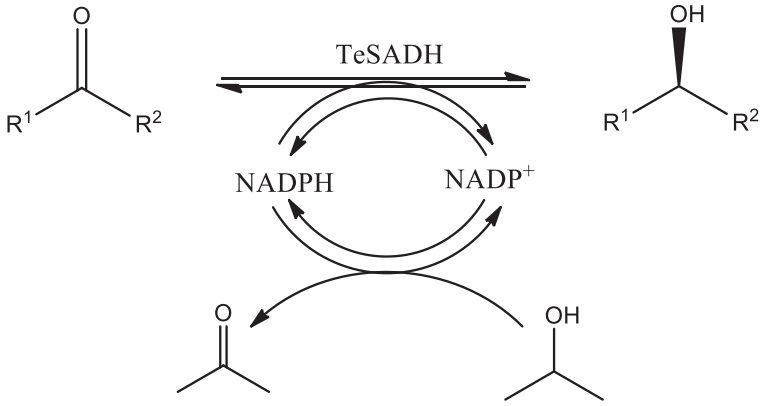


Figure 5: Regeneration of coenzyme in TeSADH-catalyzed transformations. R^1 has higher Cahn-Ingold-Prelog priority than R^2.

1.4 Asymmetric synthesis of methoxy and chloro substituted 2-tetralols and various phenyl ring containing ketones

Many bioactive compounds and heterocyclics, such as prostaglandin, dyes, steroids, and pharmaceuticals contain tetrahydronaphthalene core as a building block [20]. Methoxy substituted tetralols have been reported to possess various degrees of biological importance in terms of inhibition of tubulin assembly [21]. Moreover, enantiomerically pure (*S*)-2-tetralol and its hydroxy and alkoxy derivatives served as

intermediate the synthesis of 2-aminotetralins, which are known to possess activity toward dopamine and serotonin receptors. In addition, they have been used in the synthesis of drugs used for the treatment of many central nervous system related disorders [22]. 6-Chloro-2-tetralol serve as building block for chiral potassium channel blocker drug candidate MK-0499 (Figure 6), which can be ultimately used for the treatment of ventricular arrhythmias and mediates polarization of cardiac tissues [23]. Furthermore, it has also been used as achiral substrate in the total synthesis of anti-myocardial ischemia in drug [24]. On the other hand, (*S*)-4-(4-Hydroxyphenyl)-2-butanol, commercially known as (*S*)-rhododendrol, finds several pharmaceutical applications including as hepato-protective [25]. Moreover, rhododendrol and its glucoside have been used in skin-lightening cosmetics and as a melanin-inhibitor [26]. 4-Pheny-2-butanone constitutes a precursor for the synthesis of antihypertensive agents such as bufeniode or labetalol [27], which use to treat high blood pressure, and spasmolytics or antileptics, such as emepronium bromide [28]. Also, protease inhibitors for the treatment of acquired immunodeficiency syndrome (AIDS) such as VX-478 and SC-52151 involve 4-phenyl-2-butanol moiety (Figure 7) [29]. Enantiomerically pure α-hydroxy carbonyl compounds are important synthons in the asymmetric synthesis of natural products. Consequently, efficient methods for the construction of enantiomerically pure, or enriched, α-hydroxy carbonyl derivatives are in demand [30]. Moreover, optically pure 1, 2-diarylethanols are pharmaceutically interesting due to their applications in anti-cancer combretastatin (Figure 8) [31].

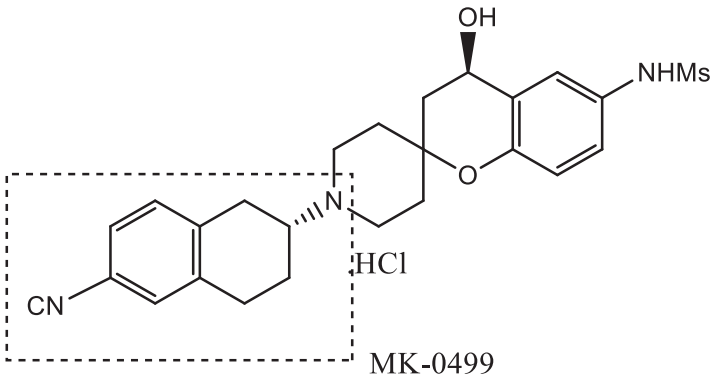
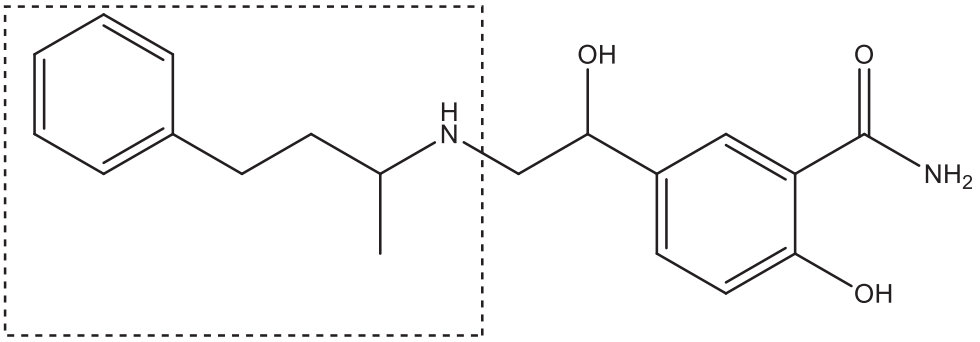
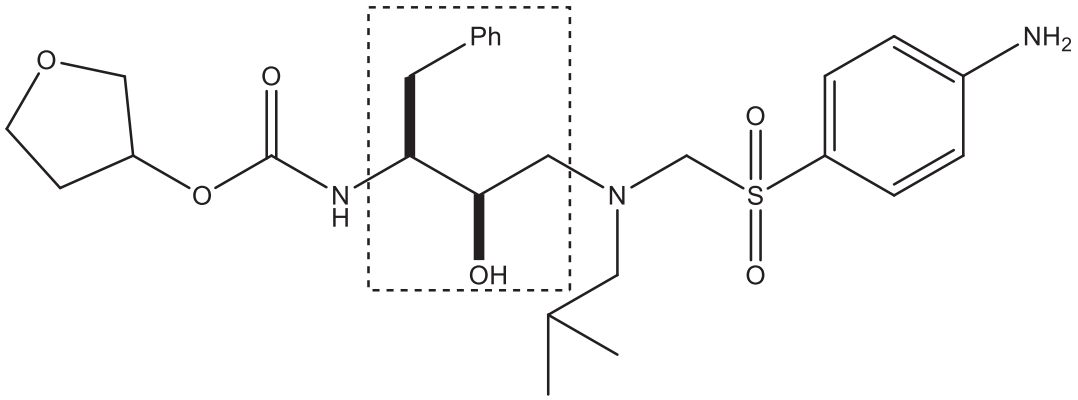


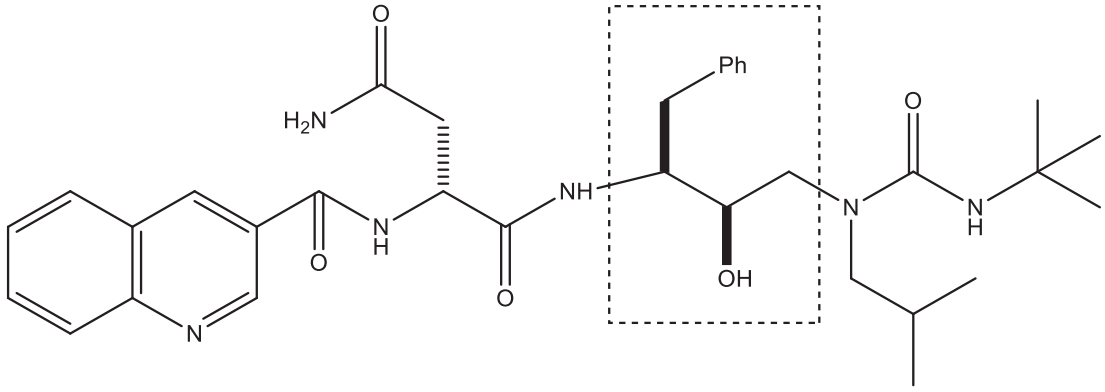
Figure 6: The pharmacological importance of tetrahydronaphthalene moiety.in various bioactive pharmscuticals.



4-phenyl-2-butanol as a bulding block in the Labetalol



VX-478 (Vertex - Glaxo Wellcome)



SC-52151 (Searle - Monsanto)

Figure 7: 4-phenyl-2-butanol scaffold containing important bioactive pharmacuticals.

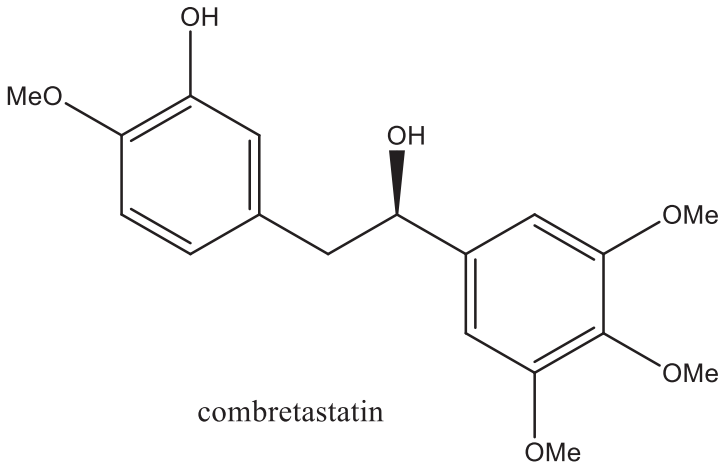


Figure 8: optically pure 1, 2-diarylethanols scaffold containing important bioactive pharmacuticals.

several synthetic methods for the asymmetric synthesis of substituted 2-tetraloles have been reported. Mogi *et al.* reported the asymmetric transfer hydrogenation of substituted 2-tetralone by $[RuCl_2$ (p-cymene)$]_2$ complex with (*R*,*R*)-N-(2-amino-1,2-diphenylethyl)-p-toluenesulfonamide or (1*S*,2*R*)-(–)-*cis*-1-amino-2-indanol (Figure 9) [32]. Likewise methoxy tetralones were also reduced to their corresponding (*R*)-configured alcohols. These reduction reactions resulted in moderate conversions (74-81%) and moderate enantioselectivities (78-88% *ee*), as shown in Table 1.

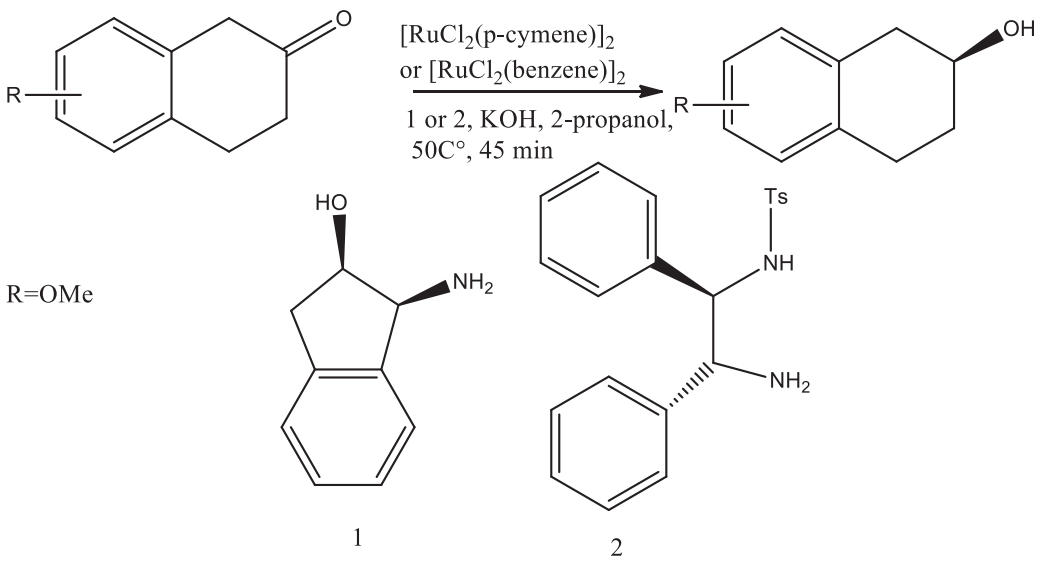


Figure 9: The asymmetric reduction of substituted methoxy of 2-tetralone using $[RuCl_2(p\text{-cymene})]_2$ and (*R*,*R*)-N-(2-amino-1,2-diphenylethyl)-p-toluenesulfonamide or (1*S*,2*R*)-(–)-*cis*-1-amino-2-indanol.

Table 1: The asymmetric reduction of 2-tetralone and its phenyl-ring-substituted analogs by transfer hydrogenation (as shown in Figure 9).

R	**Ligand**	**Yield (%)**	***ee* (%)**
H	1	81	78
5-OMe	1	80	82
6-OMe	1	85	83
7-OMe	1	81	78
8-OMe	1	74	92
H	2	87	78
5-OMe	2	82	77
6-OMe	2	83	80
7-OMe	2	81	74
8-OMe	2	80	96

On the other hand, the asymmetric reduction of 2-tetralone, 5-methoxy-2-tetralone, 7-methoxy-2-tetralone, and 8-methoxy-2-tetralone by whole cells of the fungus *Fusarium culmorum* was achieved using, which led to the formation of the corresponding (*S*)-alcohols with high conversion and moderate enantiomeric excess, (Figure 10) [33].

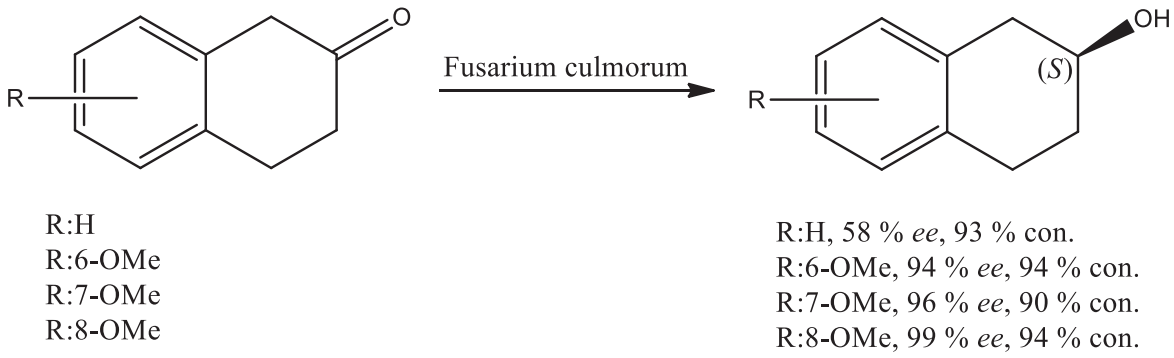


Figure 10: The asymmetric reduction of 2-tetralone and its methoxy substituted analogues by *Fusarium culmorum*.

Daucus carota and yeast cells were also used of asymmetric reduction of 2-tetralones was done by using [34]. These reactions produced the corresposning (*R*)-configured alcohols in moderate enantioselectivity (70–80% *ee*) and poor conversion (50–60%) with the long incubation periods (Figure 11).

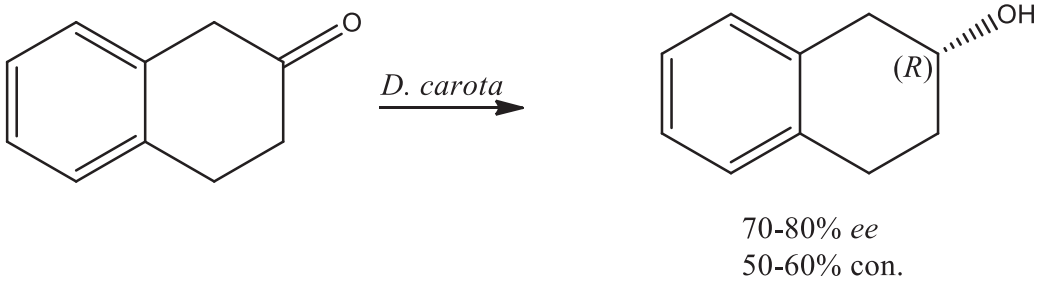


Figure 11: The asymmetric reduction of 2-tetralones by using *Daucus carota* and yeast cells.

In another attempt Asymmetric reduction of 2-tetralone accomplished by *Kluyveromyces marxianus* CBS 6556 yeast cells to produce (*S*)-2-tetralol quantitatively in 76% *ee* (Figure 12) [35].

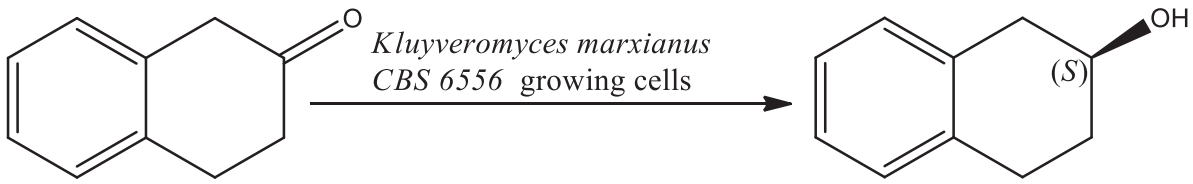
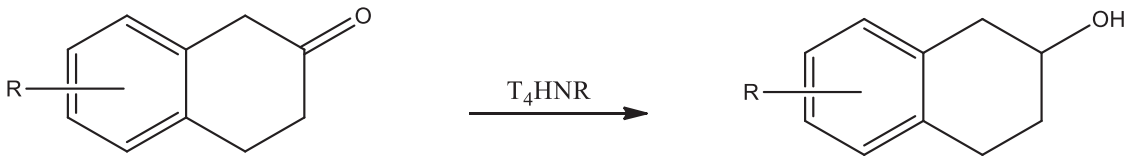


Figure 12: The asymmetric reduction of 2-tetralone by *Kluyveromyces marxianus* CBS 6556 yeast cells.

In another report the asymmetric reduction of 2-tetralone and its methoxy and hydroxyl derivatives was carried out by tetrahydroxy naphthalene reductase (T_4HNR) and T_3HNR [36]. Whereas T4HNR-catalyzed reduction of 2-tetralone to (*S*)-2-tetralol with 61% *ee* and 99% conversion, the *ee* and conversion of substituted 2-tetrlones turned to be high, as shown in (Figure 13) and Table 2. Surprisingly, the asymmetric reduction of 5-methoxy-2-teralone by this method resulted in the corresponding *R* alcohol. Docking studies of this substrate within the active site of T_4HNR showed that the inverse fit of this substrate , which is due to the hydrophobic interactions between the methyl of the methoxy group and leu-232 and Ile-274 moieties resides within the active site of T_3HNR/ T_4HNR. A comparative study T_4HNR and T_3HNR in the asymmetric reduction of 2-tetralone, 5-mehtoxy-2-tetralone, and 7-mehtoxy-2-tetralone revealed enhancement in the ee of 5-mehtoxy-2-tetralone when T_4HNR was used as a biocatalyst Table 3 [37].



R:H ,2-tetralone
R:5-OMe, 5-methoxy-2-tetralone
R:7-OMe,7-methoxy-2tetralone
R:8-OMe,8-methoxy-2-tetralone
R:6-Chloro,6-chloro-2-tetralone

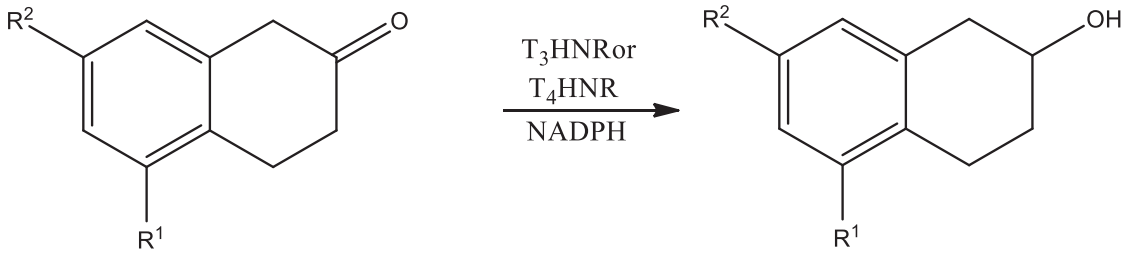
R:H ,2-tetralol
R:5-OMe, 5-methoxy-2-tetralol
R:7-OMe,7-methoxy-2tetralol
R:8-OMe,8-methoxy-2-tetralol
R:6-Chloro,6-chloro-2-tetralol

Figure 13: The asymmetric reduction of 2-tetralone and its methoxy and derivatives by tetrahydroxy naphthalene reductase (T_4HNR).

Table 2: The asymmetric reduction of 2-tetralone and its methoxy and hydroxyl derivatives by tetrahydroxy naphthalene reductase (T_4HNR).

substrate	***ee***	**conversion**	**configuration**
2-tetralone	61	99	*S*
5-methoxy-2-tetralone	99	99	*R*
7-methoxy-2-tetralone	> 99	99	*S*
8-methoxy-2-tetralone	99	99	*S*

Table 3 : Reduction of 2-tetralone (1), 5-methoxy-2-tetralone (2), and 7-methoxy-2-tetralone (3) by T3HNR/ T4HNR.



1: $R^1/R^{2=}$ H
2: R^1= OCH_3; R^2= H
3: R^1= H; R^2= OCH_3

1: R^1/R^2= H
2: R^1= OCH_3; R^2= H
3: R^1= H; R^2= OCH_3

Enzyme	Substrate	Yield (%)	*ee* (%)	Configuration
T_3HNR	1	66	60	*S*
T_3HNR	2	71	75	*R*
T_3HNR	3	67	> 99	*S*
T_4HNR	1	77	61	*S*
T_4HNR	2	57	99	*R*
T_4HNR	3	71	> 99	*S*

In their findings, Musa *et al.* has reported the enantioselective reduction of phenylacetone by W110A TeSADH to produce (*S*)-1-phenyl-2-propanol in only 37% ee. However, W110A TeSADH-catalyzed reduction of 1-(4'-methoxyphenyl)-2-propanone resulted in the corresponding (*S*)-configured alcohol in > 99% *ee* (figure 14) and 2-tetralone results in (*S*)-2-tetralol in 71% *ee*.

The significant improvement in the enantioselectivity that due to the presence of methoxy group in phenylacetone (Figure 14) encouraged us to study the asymmetric reduction of substituted tetralones in asymmetric way in order to know the effect of substituents on the enantioselectivity of the reduction reaction.

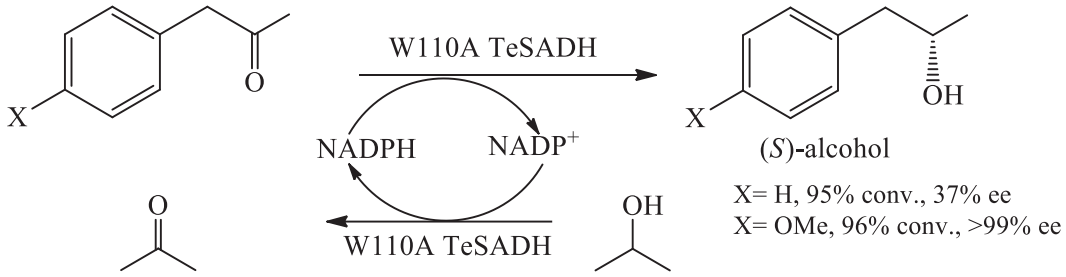
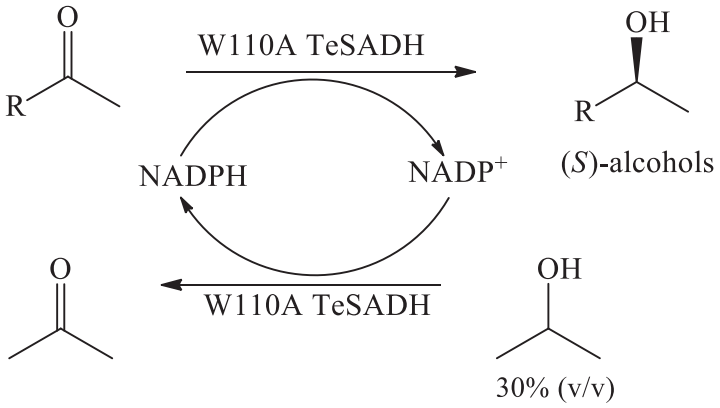


Figure 14: The asymmetric reduction of phenylacetone and 4-(4'-methoxyphenyl)-2-propanone using W110A TeSADH

Some prochiral phenyl ring containing ketones which were reduced using W110A TeSADH to produce their corresponding (*S*)-configured alcohols [19]. With the exception of phenylacetone, which was reduced with poor enantioselectivity, all other tested ketones were reduced with high enantioselectivities and good yields (Table 4).

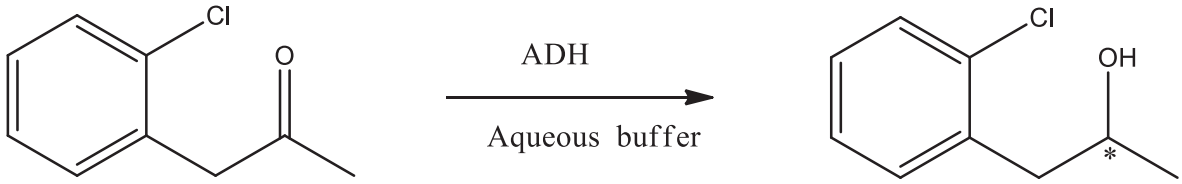
Table 4: Enantioselective reduction of phenyl-ring-containing ketones using W110A TeSADH.



R	**Conv. (%)**	***ee* (%)**
$PhCH_2CH_2$	99	> 99
$Ph(C{=}O)CH_2$	98	> 99
(*E*)-Ph-HC=CH	64	> 99
p-$MeOC_6H_4(CH_2)_2$	87	91
$PhOCH_2$	> 99	> 99
$PhCH_2$	95	37
p-$MeOC_6H_4CH_2$	97	> 99

There are fewer reports on the asymmetric reduction of 1-(2-chlorophenyl)acetone using biocatalysis. Including the use of several types of ADH table 5 [38].

Table 5: Asymmetric reduction of 1-(2-chlorophenyl)acetone by ADH



ADH	Conversion (%)	ee (%)	Configuration
LP	20	> 99	*R*
CP	97	> 99	*S*
PR_2	15	84	*R*
A	99	> 99	*S*

Although ADHs are versatile biocatalysts catalyzing a wide range of phenyl ring containing ketones, the enantioselective reduction of diaryl ketones (bulky-bulky ketones) or aryl- alkyl with sterically bulky alkyl groups such as propyl or phenyl ring containing ketones possessing two adjacent carbonyl groups, and the reduction of phenyl ring containing ketones in the anti-Prelog fashion still represents a significant challenge in both the enzymatic and chemical reductions of ketones, The highly stereoselective deduction by ADHs is preferred when ketone bears one small and a bulky groups, Moreover, the smaller group is limited to a sterically nondemanding group such as methyl, ethyl, or chloromethyl [38]. Only few methods have been reported for the biocatalytic asymmetric reduction of ketones that bear two bulky groups (bulky–bulky ketones) by using (fermenting) whole cells [39] or purified enzymes[40]. Moreover, very few ADHs have shown *anti*-Prelog stereopreference [41, 38].

Therefore, our study focuses on we report the asymmetric bioreduction of various phenyl ring containing ketones by using different mutants of TeSADH. The specific aim of this research endeavor was to expand the biocatalyst substrate specificity, as well its chemo, –regio, and enantioselectivities related to the molecular structure and to compare the effect of different mutants on enantioselectivity and steroselectivity. The kinetic parameters of these reactions are also reported.

CHAPTER 2

OBJECTIVES AND WORK PLAN

The main objective of this proposed study was to synthesize optically active substituted 2-tetralols and other phenyl-ring-containing ketones, important building blocks in pharmaceutical industries, from their corresponding ketones using TeSADH mutants. These ketones included in this study were 2-tetralone, 6-chloro-2-tetralone, 5-methoxy-2-tetralone, 7-methoxy-2-tetralone, 8-methoxy-2-tetralone, and other phenyl-ring-containing ketones like as 4-phenyl-2-butanone, 4-(4-methoxyphenyl)-2-butanone, 4-phenyl-3-butyn-2-one, 1-(2-chlorophenyl)acetone, 1-(4-chlorophenyl)acetone and 1,2-diphenylethanone.

W110V, W110A, W110G and W110A/I86A mutants of TeSADH were used as catalysts for this study. One of the major purposes of this study was to expand the substrate specificity of TeSADH by screening substrates that were never been used with this enzyme. The enzyme kinetics for these transformations were conducted to check the enzyme's efficiency towards the proposed substrates and to compare the resultants parameters (K_m and k_{cat}), with the reported substrates such as 4-phenyl-2-butanone and phenylacetone with W110A TeSADH,

The objectives of the main our study was to:

i. Expand the substrate specificity of TeSADH.

ii. Employ W110G, W110A, W110V, W110A/I86A mutants in the asymmetric reduction of different phenyl ring prochiral ketones containing and to compare the percent conversion and enantiomeric excess for their reaction products.

iii. Purify the optically active alcohols products when needed.

iv. Characterize the chemical identity of the reaction products by IR, ^{1}H and ^{13}C NMR, and GC-MS.

v. Characterize the stereochemical outcome of alcohols products by GC chiral column and polarimetry.

vi. Study enzyme kinetics for W110A and W110G TeSADH-catalyzed reduction of phenyl ring prochiral ketones containing.

Chapter 3

Experimental

3.1 General

Capillary gas chromatographic (GC) measurements were performed on a GC equipped with a flame ionization detector and an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μm film thickness) using He as the carrier gas. High pressure liquid chromatographic (HPLC) measurements were performed on a HPLC on a Chiralcel OD–H column (Daicel). Nuclear Magnetic Resonance (NMR) spectra were recorded on a 500 MHz spectrometer at 500 MHz (^{1}H) and at 125 MHz (^{13}C) at room temperature in $CDCl_3$ using the solvent peak as an internal standard. Enzyme assays to determine the enzyme kinetics were carried out using a Cary 100 UV-Vis spectrometer equipped with a temperature control from Agilent technology. Commercial grade solvents were used without further purification. NADPH, $NADP^+$, dithiothreitol (DTT), $ZnCl_2$, 2-tetralone (**1a**), 5-methoxy-2-tetralone (**1b**), 7-methoxy-2-tetralone (**1c**), 8-methoxy-2-tetralone (**1d**), 6-chloro-2-tetralone (**1e**), 4-phenyl-2-butanone (**1f**), 4-(4-methoxyphenyl)-2-butanone (**1g**), 4-(4-hydoxyphenyl)-2-butanone (**1h**), 4-phenyl-3-buten-2-one (**1i**), 4-phenyl-3-butyn-2-one (**1j**), 1-phenylpropane-2-one (**1k**), 1-(2-chlorophenyl)propan-2-one (**1l**), 1-(3-(trifluoromethyl)phenyl)propan-2-one (**1m**), 1-phenyl-2-butanone (**1n**), 1-phenyl-2-pentanone (**1o**), 1-phenylethannone (**1p**), 1,2-diphenylethanone (**1q**), 1,3-diphenyl-2-propen-1-one (**1r**), 1-phenylpropane-1,2-dione (**1s**), 1-phenylbutane-1,3-dione (**1u**), 4,4,4-trifluoro-1-phenylbutane-1,3-dione (**1v**), (*rac*)-1-phenylpropane-2-ol [(*rac*)-**2k**],

(*rac*)-1-phenylethanol [(*rac)*-**2p**], and (*rac*)-1,3-diphenyl-2-propan-1-ol [(*rac*)-**2r**] were used as purchased from commercial sources. **(***rac*)-2-Tetralol [(*rac*)-**2a**], (*rac*)-5-methoxy-2-tetralol [(*rac*)-**2b**], (*rac*)-7-methoxy-2-tetralol [(*rac*)-**2c**], (*rac*)-8-methoxy-2-tetralol [(*rac*)-**2d**], (*rac*)-6-chloro-2-tetralol [(*rac*)-**2e**], **(***rac*)-4-phenyl-2-butanol [(*rac*)-**2f**], (*rac*)-4-(4-methoxyphenyl)-2-butanol [(*rac*)-**2g**], (*rac*)-4-(4-hydoxyphenyl)-2-butanol [(*rac*)-**2h**], **(***rac*)-4-phenyl-3-buten-2-ol [(*rac*)-**2i**], (*rac*)-4-phenyl-3-butyn-2-ol [(*rac*)-**2j**], (*rac*)-1-(2-chlorophenyl)-2-propanol [(*rac*)-**2l**], **(***rac*)-1-(3-(trifluoromethyl)phenyl -2-propanol [(*rac*)-**2m**], (*rac*)-1-phenyl-2-butanol [(*rac)*-**2n**], (*rac*)-1-phenyl-2-pentanol [(*rac)*-**2o**], and **(***rac*)-1,2-diphenylethanol [(*rac*)-**2q**], were produced by reducing their corresponding ketones with $NaBH_4$ as reported [42].

3.2 Gene expression and purification of TeSADH mutants

W110A, W110G, W110V and W110A/I86A mutants of TeSADH were constructed, expressed and purified as previously reported [43].

3.3 General procedure for asymmetric reduction of substituted 2-tetralones and other phenyl ring containing ketones

3.3.1 Small scale reduction reactions

The asymmetric reduction reactions of substituted 2-tetralones and other phenyl ring containing ketones by TeSADH mutants were conducted according to a published procedure with some modifications [44,45]. The ketone (0.0191 mmol) was added to a solution containing the enzyme (0.2-0.4 mg), $NADP^+$ (1.0 mg) in Tris-HCl buffer solution (700 μL, 50 mM, pH 8.0). And 2-propanol (300 μL) in a 1.5-mL Eppendorf tube. The mixture was shaken in a thermostated shaker at 50 ºC and 180 rpm for 24 h. W110V TeSADH-catalyzed reactions were carried out by using ketones (0.00382 mmol) in Tris-HCl containing 2-propanol (95:5, v/v) followed by the addition of $ZnCl_2$ (5 μL of 0.27 mM) and DDT (1.0 mg) were added to I86A/W110A TeSADH-catalyzed reduction reactions. The enzyme loading and 2-propanol concentrations were varied to achieve maximum conversion for selected substrates. The reaction mixture was then extracted with ethyl acetate (2 × 500 μL). The combined organic layers were dried with sodium sulfate and concentrated. The remaining residue was treated with acetic anhydride (5 μL) and pyridine (10 μL) to obtain the acetate ester derivative prior to *ee* determination by chiral GC [46] .The *ee* of substrates (**2q, 2v**) were analyzed by a chiral HPLC.

3.3.2 Large-scale asymmetric reduction reactions

Reaction was carried out on 10-fold scale by using ketones (0.191 mmol) in Tris-HCl buffer solution (50 mM, pH 8.0) containing 2-propanol (10 mL, 70:30, v/v), enzyme (0.2-0.4 mg) and $NADP^+$ (2 mg). The reaction was carried out at 50 ^oC and 180 rpm in 50 mL round-bottomed flasks. The reaction mixture was then extracted with ethyl acetate (2 × 7 mL) and combined organic layers were dried with sodium sulfate, concentrated and derivatized as described previously prior to GC analysis. Whereas (**2q, 2v)** were analyzed by HPLC, (*R*)-1,2-diphenylethanol and 1,3-diphenyl-2-propan-1-ol were purified by flash chromatography using a mixture of ether:hexane (1:3, v/v), and ethyl acetate:hexane (1:1, v/v), respectively. (*R*)-8-Methoxy-2-tetralol and (*S*)-6-chloro-2-tetralol were also purified using by flash chromatography using a mixture of ether: hexane (4:1).

3.4 Characterization and Quantification of reduction Products

To confirm the chemical identity of the produced alcohols, the purified alcohols were characterized by various spectroscopic techniques including NMR (^1H and ^{13}C), IR, and mass spectrometry (MS). To identify the stereochemical identity of the produced alcohols, their optical rotation were measured by polrimetry, and were then compared with the reported values. The % *ee* of alcohols (**1q, 1v**) was determined by HPLC equipped with a chiral column, whereas for other produced alcohols were transformed to their acetate derivatives prior to their analysis by a GC equipped with a chiral column.

3.5 GC analysis (chiral)

The *ee* of ester derivatives was determined by Agilent GC model 7890A equipped with an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μm film thickness) using He as the carrier gas and using flame ionization detector (FID). The following temperature program was used for (**2a-c** , **2e, 1j, 1l, 1o**): Oven 70 ^{o}C (initial, hold time 10 min) to 220 ^{o}C (final, hold time 10 min) at 1.5 ^{o}C/min rate; Injector-220 ^{o}C, detector 230 ^{o}C, He flow rate of 15 mL/min. Injection volume was 1.0 μL with split ratio 10:1. For **2d** and the temperature program used was: Oven 70 ^{o}C (initial, hold time 10 min) to 220 ^{o}C (final, hold time 10 min) at 0.3 ^{o}C/min rate; Injector-220 ^{o}C, detector 230^{o}C, He flow rate of 15 mL/min. Injection volume was 1.0 μL with split ratio 10:1. On the other hand, the following temperature program was used for (**1f-1i, 1k, 1m, 1n, 1p, 1s, 1u**) for GC oven 70 ^{o}C (initial, hold time 10 min) to 180 ^{o}C (final, hold time 20 min) at 5 ^{o}C/min rate; injector 220 ^{o}C, detector 230 ^{o}C, Helium flow rate is 15 mL/min. Injection volume was 1.0 μL with split ratio 10:1.

3.6 HPLC analysis (chiral)

The *ee* of produced alcohol was determined (Agilent HPLC model) using hexane/methanol (v/v = 95: 5) as a mobile phase for **1q,** while for **1v** the mobile phase was hexane/2-propanol (v/v = 95: 5).

3.7 GC-MS analysis (non-chiral):

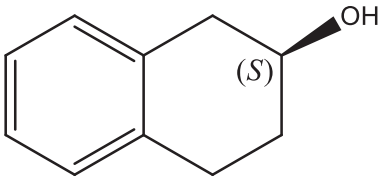
GC-MS analysis was conducted using Agilent GC model 7890A and Agilent 5975C Inert MSD with Triple-Axis Detector using capillary column [(5% phenyl)–methylpolysiloxane, 30 m × 320 µm × 0.25 µm]. The following temperature program was used for GC: Oven 70 ^{o}C (initial, hold time 10 min) to 180 ^{o}C (final, hold time 20 min) at 1.5 ^{o}C/min rate; Injector 220 ^{o}C, detector 230 ^{o}C, Helium flow rate is 15 mL/min. Injection volume was 1.0 μL with split ratio 10:1.

3.8 Enzyme kinetics

Enzyme kinetic assays were performed according to published procedures with some modifications [47, 48]. Reduction reaction rates were measured by tracking the consumption of NADPH spectrophotometrically at 340 nm ($\Delta\varepsilon$= 6220 $M^{-1}.cm^{-1}$). The kinetic assays were conducted in cuvettes containing Tris-HCl buffer solution (pH 6.5, 50 mM), NADPH (0.25 mM), CH_3CN (10%, v/v), and ketone substrate (0.05-15 mM) in a total volume of 1.2 mL at 50 °C. The cuvettes were incubated for about 15 minutes at 50 °C prior to the addition of enzyme, which was also pre-incubated separately at the same temperature. To determine the kinetic parameters, activity was determined with at least six substrate concentrations in the range of (0.001-15 mM) in triplicate. The K_m and V_{max} were calculated using Origin .

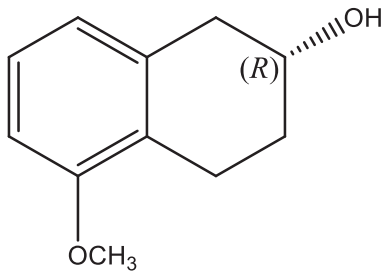
3.9 Characterization of Products

(S)-2-Tetralol [(*S*)-2a]



Pale yellow oil $[\alpha]_D^{20} = -$ 17.7° (*c* 0.011, EtOH), 69% ee, [lit. [49] $[\alpha]_D^{25} = -$ 20.8° (*c* 0.25, MeOH, 29 % ee (*S*)]; ^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.69-1.82 (m, 2H), 2.03 (s, 1H), 2.73-2.96 (m, 3H), 3.08 (dd, *J* = 3.9, 15.6 Hz, 1H), 4.15-4.23 (m, 1H), 7.09-7.24 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 27.0, 31.5, 38.4, 67.2, 125.8, 125.7, 128.6, 129.5, 134.2, 135.6; RT-IR (neat) 3339 (br), 3060 (m), 3018 (m), 2926 (m), (m) cm^{-1}; EI MS m/z (relative intensity) 148 (M^+] (12), 131 (12), 130 (100), 129 (42), 128 (12), 115 (27), 105 (14), 104 (59), 103 (18), 91 (16), 78 (14), 77 (10).

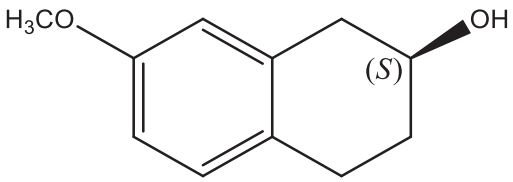
(*R*)-5-Methoxy-2-tetralol [(*R*)-2b]



Pale yellow oil $[\alpha]_D^{20} = +$ 12.5° (*c* 0.008, EtOH), 61 % ee, [lit [50] $[\alpha]_D^{27} = +$ 57° (*c* 0.25, EtOH, 88 % ee (*R*)]; ^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.78-1.87 (m, 2H), 2.04-2.14 (m, 1H), 2.62-2.74 (m, 1H), 2.93 (dt, *J* = 4.8, 18.9 Hz, 1H), 3.07 (dd, *J* = 4.3, 16.2 Hz, 1H), 3.81 (s, 3H), 4.12-4.18 (m, 1H), 6.66 (d, *J* = 8.2 Hz, 1H), 6.70 (d, *J* = 7.4 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 21.2, 31.0, 38.4, 55.2, 67.0, 107.2,

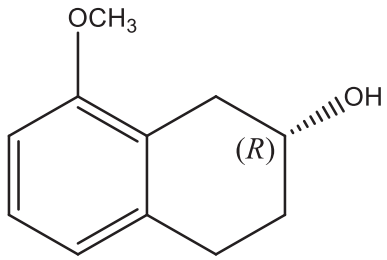
121.6, 124.5, 126.4, 135.6, 157.1; FT-IR (neat) 3060 (m), 3018 (m), 2926 (m), 2840 (m) cm^{-1}; EI MS m/z (relative intensity) 178 [M^+] (76), 160 (100), 145 (57), 134 (35), 104 (53), 91 (42), 51 (6).

(*S*)-7-Methoxy-2-tetralol [(*S*)-2c]



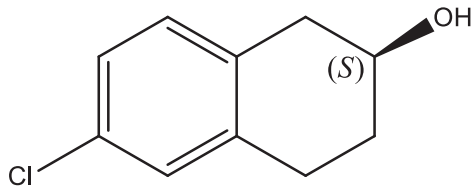
Pale yellow oil $[\alpha]_D^{20} = -20.4°$ (*c* 0.0069, EtOH) (> 99 % ee), (lit. [50] $[\alpha]_D^{27} = +45°$ (*c* = 0.22, EtOH, 88 % ee (*R*)); ^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.79-1.90 (m, 2H), 2.01-2.14 (m, 1H), 2.70-2.75 (m, 2H), 2.85-2.88 (m, 1H), 3.03 (dd, *J* = 4.0, 16.2 Hz, 1H), 3.75 (s, 3H), 4.10-4.19 (m, 1H), 6.60 (s, 1H), 6.68 (dd, *J* = 1.8, 8.6 Hz, 1H), 6.99 (t, *J* = 8.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 26.1, 31.7, 38.65, 55.3, 67.3, 112.0, 114.0, 127.7, 129.5, 135.4, 157.8; RT-IR (neat) 3060 (m), 3018 (m), 2926 (m), 2840 (m) cm^{-1}; EI MS m/z (relative intensity) 178 (76) [M^+], 160 (100), 145 (57), 134 (35), 104 (53), 91 (42), 51 (6).

(*R*)-8-Methoxy-2-tetralol [(*S*)-2d]



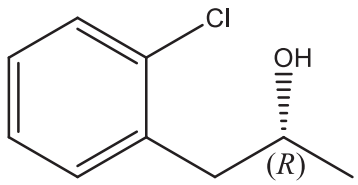
Pale yellow oil $[\alpha]_D^{20}$ = + 52.41° (*c* 0.00145, EtOH) (> 99 % ee), (lit. [50] $[\alpha]_D^{27}$ = + 48.6° (*c* = 0.27, EtOH, 98 % ee (*R*)) ; ^{1}H NMR (500 MHz, $CDCl_3$) : δ (ppm) 1.60-1.63 (m, 1H), 1.79-1.81 (m, 1H), 1.99-2.02 (m, 1H), 2.55 (dd, *J* = 7.6, 17.1 Hz, 1H), 2.82-2.85 (m, 1H), 2.94 (dt, *J* = 5.8, 17.1 Hz, 1H), 3.08 (dd, *J* = 5.1, 17.4 Hz, 1H), 3.81 (s, 3H), 3.99-4.17 (m, 1H), 6.67 (d, *J* = 8.2 Hz, 1H), 6.72 (d, *J* = 7.9 Hz, 1H), 7.09 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 27.1, 31.1, 32.6, 55.4, 67.4, 107.1, 120.8, 123, 126.3, 137.2, 157.53; FT-IR (neat) 3060 (m), 3018 (m), 2926 (m), 2840 (m) cm^{-1}; EI MS m/z (relative intensity) 178 [M^+] (59), 160 (78), 145 (43), 134 (64), 129 (38), 115 (53), 104 (100), 91 (93), 77 (40), 65 (33), 51 (21).

(*S*)-6-Chloro-2-tetralol [(*S*)-2e]



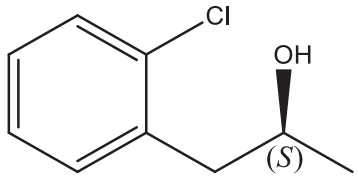
Pale yellow oil $[\alpha]_D^{20}$ = − 43° (*c* 0.002, $CHCl_3$) (> 99 % ee), (lit. [51] $[\alpha]_D^{15}$ = − 20.8° (c 1.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) : δ 1.76-1.84 (m, 1H), 1.99-2.05 (m, 1H), 2.71 (dd, *J* = 7.6, 16.4 Hz, 1H), 2.76-2.83 (m, 1H), 2.94 (dt, *J*= 6.1, 17.1 Hz, 1H), 3.03 (dd, *J* = 4.8, 16.6 Hz, 1H), 4.15-4.18 (m, 1H), 6.99-7.09 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$)

: δ 26.6, 31.0, 37.8, 66.9, 126.0, 128.3, 132.6, 130.3, 137.5, 131.5; FT-IR (neat) 3339 (br), 3060 (m), 3018 (m), 2926 (m), 2840 cm^{-1}; EI MS m/z (relative intensity) 182 (20) [M^+], 164 (43), 138 (57), 129 (100), 115 (25), 103 (43).



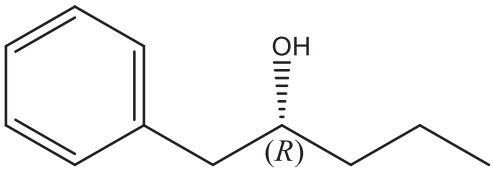
(*R*)-1-(2-Chlorophenyl)propan-2-ol [(*R*)-2l]: $[\alpha]_D^{21}$ = – 20° (*c* 0.011, CH_2Cl_2) 80 % *ee*, lit.[52] $[\alpha]_D^{20}$ = + 40.3 (*c* 1.0, CH_2Cl_2), 95% *ee* for *S* isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (d, *J* = 6.1 Hz, 3H), 1.54 (br s, 1H, OH), 2.83 (dd, *J* = 7.9, 13.7 Hz, 1H), 2.96 (dd, *J* = 4.6, 13.6 Hz, 1H), 4.12-4.16 (m, 1H), 7.16-7.38 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.0, 43.2, 67.5, 126.7, 127.9, 129.6, 131.7, 134.3, 136.4. RT-IR (neat) 3364 (m), 3057 (m), 2917 (m) cm^{-1}. EI MS m/z (relative intensity) 155 (8), 126 (85), 91 (100), 66 (15), 45 (50).

(*S*)-1-(2-chlorophenyl) propan-2-ol [(*S*)-2l]



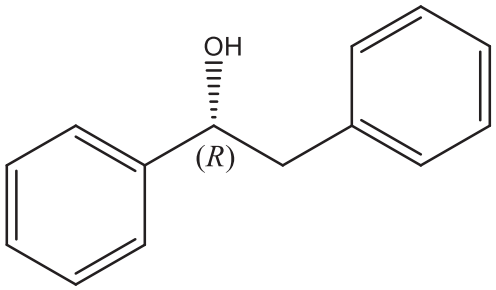
(*S*)-1-(2-Chlorophenyl) propan-2-ol [(*S*)-2g]: $[\alpha]_D^{22}$ = + 11° (*c* 0.009, CH_2Cl_2) 98 % *ee*, lit. [52] $[\alpha]_D^{20}$ = + 40.3 (*c* 1, CH_2Cl_2), 95% *ee* for *S* isomer.

(*R*)- 1-phenylpentan-2-ol [(*R*)-2o]



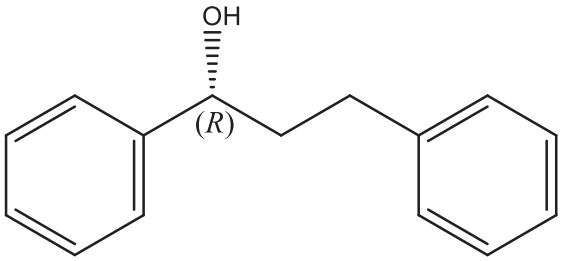
$[\alpha]_D^{21}$ = – 1.01 ° (*c* 0.015, EtOH) (61.5 % ee), [lit. [53] $[\alpha]_D^{20}$ = + 3.7° (*c* 1.1, EtOH, (*S*)] 99% *ee* for *S* isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.92-0.95 (t, *J* = 7.0 Hz, 3H), 1.41-1.53 (m, 4H), 2.65 (dd, *J* = 8.5, 13.6 Hz, 1H), 2.83 (dd, *J* = 13.6, 4.0 Hz, 1H), 3.67 (br s, OH), 3.81-3.86 (m, 1H), 7.19-7.35 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ 44, 72, 39, 19, 14, 138, 129, 128, 126. RT-IR (neat) 3347 (m), 3018 (m), 2920 (m) cm^{-1}. EI MS m/z (relative intensity) 121 (8), 92 (100), 55 (21), 32 (91).

(*R*)-1, 2-diphenylethanol [(*R*)-2q]



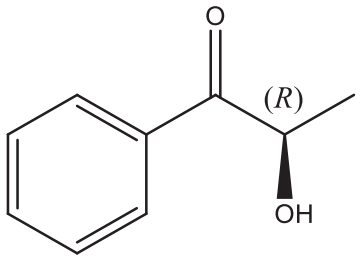
$[\alpha]_D^{24}$ = + 7.4° (*c* 0.0157, $CHCl_3$) (> 99 % ee), lit. [54] $[\alpha]_D^{22}$ = – 13.0 (c = 1.049, $CHCl_3$), 90% *ee* for *S* isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.93 (br s, 1H), 2.99 (dd, *J* = 8.6, 13.7 Hz, 1H), 3.05 (dd, *J* = 4.6, 13.6 Hz, 1H), 4.88-4.92 (m, 1H), 7.19–7.37 (m, 10H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 46.0, 75.0, 125.8, 126.6, 127.6, 128.4, 128.5, 129.5, 138.0, 144.0; FT-IR (neat) 3404 (br), 3084 (w), 3062 (w), 2924 (m) cm^{-1}. EI MS m/z (relative intensity) 198 (2) [M], 107 (72), 92 (100), 77 (56), 51 (12), 39 (8).

(*R*)-1, 3-diphenylpropan-1-ol [(*R*)-2r]



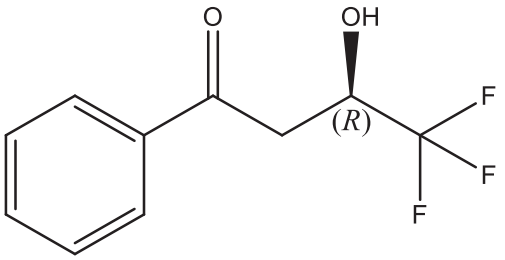
$[\alpha]_D^{21}$ = + 5° (*c* 0.005, $CHCl_3$), lit. [55] $[\alpha]_D^{20}$ = – 23.6 (*c* 0.64, CH_2Cl_2) 96% *ee* for *S* isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.46–2.99 (m, 2H), 3.50–3.58 (m, 3H),), 4.55-4.59 (m, 1H), 7.22–7.56 (m, 8H) 7.88-7.95 (m, 2H), 7.39-7.42 (d, *J*= 4.2 Hz, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 35.5, 45.4, 72.0, 127.5, 127.8, 127.9, 128.0, 128.6, 128.7, 133.4, 137.0. FT-IR (neat) 3385 (br), 3053 (w), 3024 (w), 2917 (m) cm^{-1}. EI MS m/z (relative intensity) 207 (100), 179 (22), 161 (11), 131 (33), 103 (33), 77 (66), 51 (27).

(*R*)-2-hydroxy-1-phenylpropan-1-one [(*R*)-2s]



Pale yellow oil $[\alpha]_D^{21}$ = – 4.8° (*c* 0.0075, CHCl3) (> 99 % ee), [lit. [56] $[\alpha]_D^{20}$ = – 67.0 (c 0.4, CHCl3), > 99% *ee* for *R* isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.46 (d, *J* = 7.0 Hz, 3H), 3.7 (s, 1H), 5.17 (q, *J* = 7.6 Hz, 1H), 7.37 (d, *J* = 6.7 Hz, 1H), 7.51 (t, *J* = 7.6 Hz, 1H), 7.62 (t, *J* = 5.0 Hz, 1H), 7.93 (d, *J* = 7.6 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 22.3, 69.3, 128.7, 128.9, 133.4, 134.0, 202.4. FT-IR (neat) 3334 (m), 2954 (w), 2923 cm^{-1}. EI MS m/z (relative intensity) 105 (100), 77 (50), 51 (14), 43 (11).

(*R*)-4, 4, 4-trifluoro-3-hydroxy-1-phenylbutan-1-one [(*R*)-2v]



$[\alpha]_D^{20}$ = + 0.69° (*c* 0.0115, MeOH) (> 99 % ee), [lit. [57] $[\alpha]_D^{15}$ = + 1.9° (84% ee (R)), c = 1.5, MeOH)] , 84% *ee* for *R* isomer. ^{1}H NMR (500 MHz, $CDCl_3$) : δ 3.2 (dd, *J* = 2.7, 18.0, Hz, 1H), 3.39 (dd, *J* = 9.1, 16.9 Hz, 1H), 3.46 (s, 1H, OH), 4.67-4.72 (m, 1H), 7.51 (t, *J* = 7.3 Hz, 3H), 7.63 (t, *J* = 7.3 Hz, 3H), 7.97 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 38.3, 128.2, 128.9, 134.2, 136.0, 197.6. FT-IR (neat) 3381 (br), 2958 (m), 2924 (m) cm^{-1}. EI MS m/z (relative intensity) 219 (4) [M], 198 (10), 105 (100), 77 (50), 51 (17).

CHAPTER 4
RESULTS AND DISCUSSION

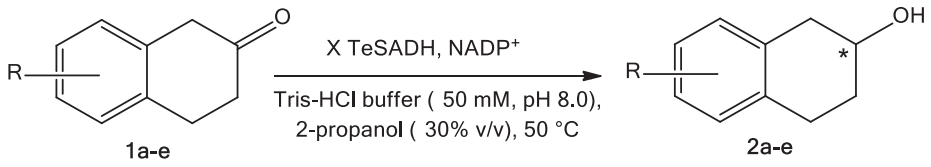
4.1 Asymmetric reduction of substituted 2-tetralones

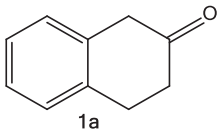
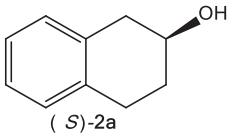
Enantioselective reductions of substituted 2-tetralones were carried out using various mutants of TeSADH (W110G, W110A, W110V, and W110A/I86A) to produce the corresponding enantiopure alcohols with high conversions and optical purities. The reactions were carried out in Tris-HCl buffer solution containing 2-propanol (30%, v/v), which serves both as co-solvent and co-substrate to regenerate the co-enzyme making the process catalytic. The use of high concentration 2-propanol was crucial to enhance the solubility of substituted 2-tetralone substrates and to shift the equilibrium of the reaction towards the product. We have reported earlier that the asymmetric reduction of phenyl-ring-containing ketones using W110A TeSADH produces the corresponding *S* alcohols, whereby the NADPH cofactor transfers its pro-*R* hydride to the *re* face of the ketone (i.e., Prelog mode). Thus, the asymmetric reduction of 2-tetralone (**1a**) using W110A TeSADH produced (*S*)-2-tetralol [(*S*)-**2a**] in 71% *ee* [16]. The low *ee* could be attributed to the small size of 2-tetralone that allows this substrate to fit in more than one orientation allowing selectivity mistakes to occur. This moderate enantioselectivity of **1a** encouraged us to explore the asymmetric reduction of **1a** and its substituted analogs (**1b-d**), using various mutants of TeSADH. It was intended to study the effect of the substituent of the phenyl ring of 2-tetralone on the enantioselectivity of the reduction reaction.

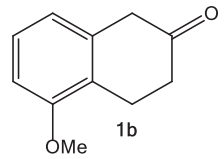
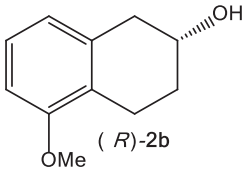
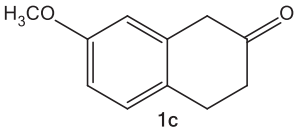
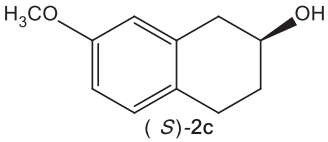
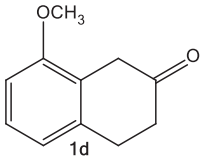
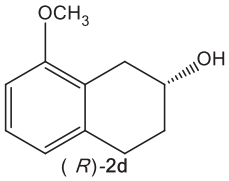
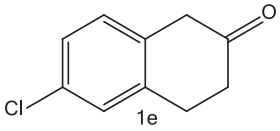
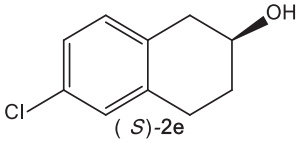
Therefore, we tried the asymmetric reduction of **1a** by various mutants of TeSADH and the enantioselectivity and the conversion of these reactions are reported in (Table 6). A moderate enantioselectivity and high percent conversion were obtained with

W110G TeSADH; these results are similar to those obtained previously with W110A TeSADH [16]. A noticeable increase in enantioselectivity was observed with W110V TeSADH, but with low percent conversion; this higher enantioselectivity may be due to the smaller size of the large pocket of W110V TeSADH compared to W110G and W110A TeSADHs, that signifies the impact on enantioselectivity by a small alteration in the active site of TeSADH. A similar trend in the enantiospecific oxidation of 1-phenyl-2-propanol by W110V TeSADH compared to W110G and W110A TeSADHs was observed previously [46]. The use of W110A/I86A TeSADH, which was designed to enlarge both pockets of TeSADH, in asymmetric reduction of **1a** resulted in moderate enantioselectivity and low conversion. The resultant alcohol has *S* configuration with the four mutant enzymes.

Table 6: Small-scale asymmetric reduction of substituted 2-tetralones by different mutants of TeSADH.[a]



Substrate	Product	Enzyme (mutant TeSADH)	Conv. (%)[b]	*ee* (%)[c]
 1a	 (*S*)-2a	W110G	> 99	69
		W110A[d]	> 99	71
		W110V	5	91
		W110A/I86A	12	74

 1b	 (*R*)-2b	W110G	> 99	61
		W110A	99	87
		W110V	96	71
		W110A/I86A	> 99	95
 1c	 (*S*)-2c	W110G	> 99	> 99
		W110A	99	> 99
		W110V	46	> 99
		W110A/I86A	> 99	> 99
 1d	 (*R*)-2d	W110G	> 99	> 99
		W110A	99	> 99
		W110V	67	> 99
		W110A/I86A	94	> 99
 1e	 (*S*)-2e	W110G	> 99	> 99
		W110A	99	> 99
		W110V	86	> 99
		W110A/I86A	95	> 99

[a] Unless otherwise stated, reactions were performed using ketone [**1a**–**e** (0.0191 mmol)], TeSADH mutant (0.2 mg), $NADP^+$ (1.0 mg), Tris-HCl buffer solution (700 μL, 50 mM, pH 8.0), and 2-propanol (300 μL); for W110A/I86A TeSADH-catalyzed reduction reaction, $ZnCl_2$ (5 μL of 0.27 mM) and DTT (1.0 mg) were also added; for W110V TeSADH-catalyzed reduction reactions, [**1a**–**e** (0.0038 mmol)], Tris-HCl buffer solution (950 μL, 50 mM, pH 8.0), and 2-propanol (50 μL) were used. [b] Percent conversion was determined by GC. [c] The % ee of the corresponding acetate ester derivative of the alcohol product was determined by GC using a chiral stationary phase. [d] [16].

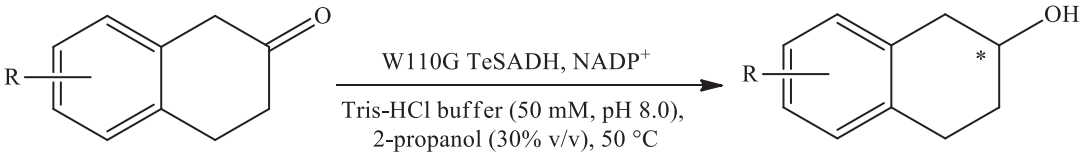
The enantioselective reduction of 5-methoxy-2-tetralone (**1b**) using W110G, W110A, W110V, and W110A/I86A TeSADHs resulted in the formation of (*R*)-5-methoxy-2-tetralol [(*R*)-**2b**] with high percent conversion and moderate to high *ee*, as shown in (Table 6). Whereas W110G TeSADH and W110A/I86A TeSADH turned out to be least and most selective mutants respectively, the W110V TeSADH and W110A TeSADH were ranged moderate in selectivity. These results demonstrate that even a small alteration in the active site of the enzyme significantly affects its enantioselectivity. In all cases, the resultant alcohol has the *R* configuration, which revealed that the presence of the methoxy group dictates the fit of **1b** in the active site. In contrast to **1a**, the fit of **1b** was in opposite fashion i.e., the hydride delivery to the *si* face of the substrate rather than to the *re* face.

The asymmetric reduction of 7-methoxy-2-tetralone (**1c**) using W110G, W110A, and W110A /I86A TeSADHs resulted in the formation of 7-methoxy-2-tetralol [(*S*)-**2c**] with high enantioselectivity and percent conversion. Reaction with W110V TeSADH produced the same (*S*)-**2c** in high enantioselectivity, but a moderate percent conversion, possibly due to the smaller large pocket of this mutant compared to the other mutants. In all cases, the resultant alcohol has the *S* configuration, which is in agreement with Prelog rule. The asymmetric reduction of 8-methoxy-2-tetralone (**1d**) produced (*R*)-8-methoxy-2-tetralol [(*R*)-**2d**] with a similar fashion as was the with **1c**, but with opposite stereopreference. The asymmetric reduction of 6-chloro-2-tetralone (**1e**) with W110G, W110A, W110V and W110A/I86A mutants of TeSADH produced the corresponding *S* alcohols in high enantioselectivities and high percent conversions. The inverted stereoselectivity noticed in the enantioselective reductions of **1b** and **1d** showed the great

influence of the position of the substituent on the aromatic ring that dictates the binding mode of the substrate in the active site, and hence the stereochemical outcome.

The same reduction was carried out in a larger scale using W110G TeSADH, since this mutant gave the highest percent conversion of the four mutant enzymes (Table 7). Slightly lower percent conversions were obtained in the large-scale reduction reactions of **1d** (95 %) and **1e** (93 %) compered to their small scale reactions, possibly due to product inhibition. However, for the other substrates the percent conversion and % *ee* were almost identical compared to small scale reactions.

Table 7: Large-scale asymmetric reduction of substituted 2-tetralones by W110G TeSADH.[a]



1a: R= H
1b: R= 5-OCH_3
1c: R= 7-OCH_3
1d: R= 8-OCH_3
1e: R= 6-Cl

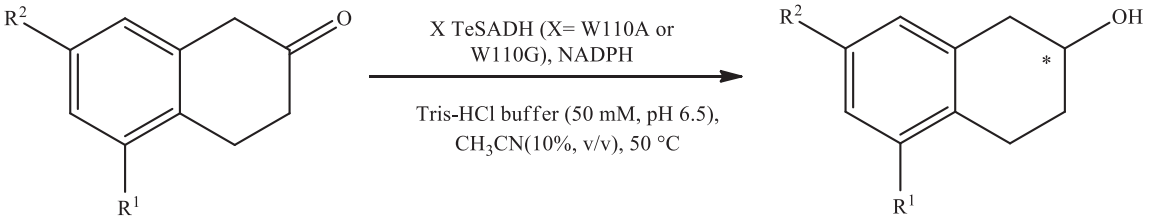
(*S*)-**2a**: R= H
(*R*)-**2b**: R= 5-OCH_3
(*S*)-**2c**: R= 7-OCH_3
(*R*)-**2d**: R= 8-OCH_3
((*S*)-**2e**: R= 6-Cl

Substrate	Product	Conv. (%)[b]	*ee* (%)[c]
1a	(*S*)-**2a**	> 99	69
1b	(*R*)-**2b**	> 99	61
1c	(*S*)-**2c**	> 99	> 99
1d	(*R*)-**2d**	95	> 99
1e	(*S*)-**2e**	93	> 99

[a] Unless otherwise stated, reactions were performed by using ketone [(1**a**–**e** (0.191 mmol)], W110G TeSADH (0.2 mg), $NADP^+$ (1.5 mg), and 2-propanol (30%, v/v). [b] % Conversion was determined by GC. [c] The % *ee* of the corresponding acetate ester derivative of the alcohol product was determined by GC using a chiral stationary phase.

To study the effect of the substituent on the aromatic ring of the 2-tetralone on the binding affinity and maximum rate, we measured Michaelis-Menten constant (K_m) and maximum rate (V_{max}) of W110A and W110G TeSADH for substrates **1a-c** (Table 8). The lowest K_m values of W110G and W110A TeSADHs were obtained for **1a**. Thus, one would expect that **1a** may be reduced with high enantioselectivity by these two mutant enzymes, which was not the case in reality (Table 6). The fact that due to the light there is little difference in the size of the two alkyl sides of the carbonyl of **1a** would allow for more than one binding mode that increased the chances for selectivity mistakes. It is predominant to note that the K_m of W110A TeSADH for three substrates is almost three times lower than that of W110G TeSADH. The smaller size of the large pocket of W110A TeSADH than that of W110G TeSADH could better guide the proper fit of these substrates within the active site of W110A TeSADH. These findings indicated the importance of residue 110 in substrate binding through Van der Waals interactions, as suggested in the crystal structure of TbADH-2-butanol binary complex [12,58] The low K_m values of W110A TeSADH for **1a-c** were supported by the higher observed *ee* values in the asymmetric reduction reactions of **1a** and **1b,** using the same mutant as compared with those obtained with W110G TeSADH (Table 1). Addition of a methoxy group to the aromatic ring resulted in higher K_m values than for **1a** (1.3-fold increase for **1b** vs. 2.5-fold increase for **1c**). The V_{max} values for **1a-c** with both mutants were comparable to each other, which indicated that the methoxy substituent does not have a pronounce effect on V_{max}.

Table 8: Kinetic parameters of W110G and W110A TeSADH for asymmetric reduction of 1a-c.[a]



1a: R^1/R^2= H
1b: R^1= OCH_3, R^2= H
1c: R^1= H, R^2= OCH_3

(*S*)-**2a**: R^1/R^2= H
(*R*)-**2b**: R^1= OCH_3; R^2= H
(*S*)-**2c**: R^1= H; R^2= OCH_3

Ketone	W110G TeSADH		W110A TeSADH	
	K_m (mM)	V_{max} (mM.min^{-1})	K_m (mM)	V_{max} (mM.min^{-1})
1a	0.0184 ± 0.000824	0.0748 ± 0.00335	0.00580 ± 0.00029	0.0589 ± 0.0048
1b	0.0231 ± 0.0011	0.0666 ± 0.00316	0.0106 ± 0.000528	0.0695 ± 0.0039
1c	0.0452 ± 0.00156	0.0743 ± 0.00257	0.0122 ± 0.000611	0.0545 ± 0.00218

[a] Unless otherwise stated, reactions were performed at 50 °C by using ketone [1**a**–**c** (0.001-15 mM)], TeSADH mutant (0.002 mg), NADPH (0.25 mM), and acetonitrile (10%, v/v).

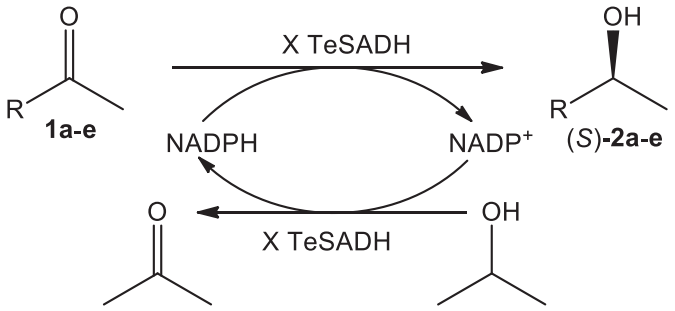
4.2 Asymmetric reduction of other phenyl-ring-containing ketones

To check the efficiency of W110A/I86A TeSADH in asymmetric reduction of phenyl-ring-containing ketones that served as substrates for W110A TeSADH [47], we conducted an extensive study on the activity and stereoselectivity of various W110 mutants (W110A, W110G and W110V) in asymmetric reduction of these ketones and compared them to those obtained by W110A/I86A TeSADH. Most of the reactions were performed in Tris-HCl buffer solutions containing 2-propanol (30%, v/v), which works as a co-solvent and co-substrate. The four mutants tested have showen similar activities and enantioselectivities in the asymmetric reduction of 4-phenyl-2-butanone (**1f**) and

produced the corresponding (*S*)-4-phenyl-2-butanol [(*S*)-**2f**] in high coversions and enantioselectivities (Table 9). The single mutants (W110A, W110G and W110V) performed slightly better than the double mutant (W110A/I86A) in the asymmetric reductions of 4-(4'-methoxyphenyl)-2-butanone (**1g**) and 4-(4'-hydoxyphenyl)-2-butanone (**1h**). However, W110A/I86A TeSADH showed the best performance and higher stereoselectivity than the single mutants in the asymmetric reduction of **1g** (Table 9). The W110G TeSADH mutant showed the least stereoselectivity in the reduction of **1g**, which is consistent with previously reported results [47]. It is well noted that the activity and stereoselectivity of TeSADH-catalyzed reduction reactions are highly dependent on the structures of both the mutant and the substrate. The high *ee* (> 94%) registered in the asymmetric reduction of **1h** using all mutants when compared with **1g** could be due to better binding affinity of hydroxyl group with the active site of the enzyme compared to methoxy group, that led to less selectivity mistakes. Low conversion was observed in the W110A/I86A TeSADH-catalyzed reduction of 4-phenyl-3-buten-2-one (**1i**) compared to those obtained with single mutants (Table 9). However, the asymmetric reduction of 4-phenyl-3-butyn-2-one (**1j**) resulted in high conversion and high *ee* with all mutants (Table 9). The asymmetric reduction of **1j** in a high enantioselectivity is of great interest since this substrate, known as a difficult substrate, which is difficult to be reduced with high enantioselectivity [59]. During optimization of the co-substrate concentration we observed an increase in the stereoselectivity in the asymmetric reduction of **1g** and **1h** by employing 10% (v/v) of 2-propanol, as compared to 30% (v/v) for **1f**, **1i** and **1j**. This outcome could be attributed to the intermolecular hydrogen bonding between the substrate and co-substrate. All of these substrates (**1f-j**) were reduced in Prelog mode by

the four mutants tested, leading to the produced alcohols in *S* configuration. The ability of W110A/I86A TeSADH to reduce these substrates with similar efficiencies to those obtained by the single mutants is charming. It is worth mentioning that W110A/I86A TeSADH was first designed to expand the two pockets of the active site as part of our effort to increase selectivity mistakes and thus increase its efficiency in racemization of enantiopure alcohols [43].

Table 9: Asymmetric reduction of phenyl-ring-containing ketones by different mutants of TeSADH.[a]

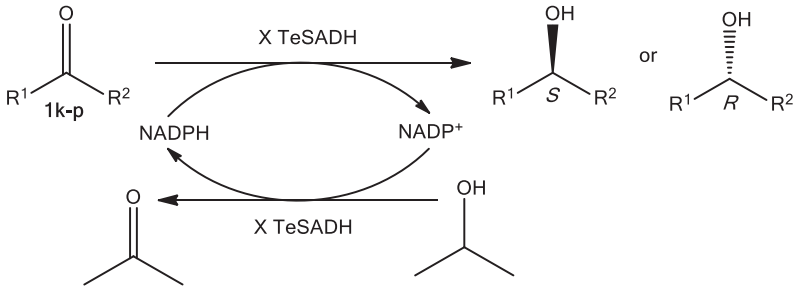


R	Substrate	X	Conv. (%)[b]	*ee* (%)[c]
$PhCH_2CH_2$	**1f**	W110G[e]	> 99	96
		W110A[e]	99	99
		W110V[e]	95	> 99
		W110A/ I86A	91	98
p-MeO-C_6H_4-CH_2CH_2	**1g**[d]	W110G	93	20
		W110A[f]	98	60
		W110V	94	79
		W110A I86A	40	84
p-HO-C_6H_4-CH_2CH_2	**1h**[d]	W110G	95	99
		W110A	97	> 99
		W110V	92	94
		W110A I86A	80	> 99
(*E*)-Ph-HC=CH	**1i**	W110G	75	> 99

		W110A[f]	73	92
		W110V	67	92
		I86A/W110A	21	90
Ph-C≡C	**1j**	W110G	> 99	97
		W110A	99	99
		W110V	97	> 99
		I86A/W110A	> 99	98

[a]Unless otherwise stated, reactions were performed using ketone **1f–j** (0.0191 mmol), TeSADH mutant [0.2 mg in 100 μL of Tis-HCl buffer solution (20 mM, pH 8.0)], $NADP^+$ (1.0 mg), Tris-HCl buffer solution (700 μL, 50 mM, pH 8.0), and 2-propanol (300 μL); for W110A/I86A TeSADH-catalyzed reduction reactions, $ZnCl_2$ (5 μL of 0.27 mM) and DTT (1.0 mg) were also added; for W110V TeSADH-catalyzed reduction reactions, **1f–j** (0.0038 mmol), Tris-HCl buffer solution (950 μL, 50 mM, pH 8.0), and 2-propanol (50 μL) were used. [b]Percent conversion was determined by GC. [c]The % *ee* of the corresponding acetate ester derivative of the alcohol produced was determined by GC using a chiral stationary phase. [d]Reactions were performed using 10% of 2-propanol. [e]Results from Patel *et al. Org. Biomol. Chem.* **2014**, *12* (31), 5905–5910. [f]Results from Musa *et al. J. Org. Chem.* **2007**, *72* (2), 30–34.

Table 10: Asymmetric reduction of phenyl ring-containing ketones by various mutants of TeSADH.[a]



R^1	R^2	Substrate	X	Conv. (%)[b]	*ee* (%)[c]
$PhCH_2$	CH_3	**1k**	W110G[d]	> 99	79(*S*)
			W110A[d]	95	84(*S*)
			W110V[d]	> 99	> 99(*S*)
			W110A/I86A	> 99	80(*R*)
o-Cl-C_6H_4-CH_2	CH_3	**1l**	W110G	13	89(*S*)
			W110A	84	98(*S*)
			W110V	99	> 99(*S*)
			W110A/I86A	> 99	99(*R*)
m-CF_3-C_6H_4-CH_2	CH_3	**1m**	W110G	> 99	96(*S*)
			W110A	> 99	>

					99(*S*)
			W110V	99	99(*S*)
			W110A/I86A	> 99	95(*S*)
$PhCH_2$	CH_3CH_2	**1n**	W110G[d]	96	92(*S*)
			W110A	98	92(*S*)
			W110V[d]	99	> 99(*S*)
			W110A/I86A	72	71(*S*)
$PhCH_2$	$CH_3CH_2CH_2$	**1o**	W110G	n.r.	-
			W110A	3	n.d.
			W110V	32	92(*R*)
			W110A/ I86A	95	98(*R*)
Ph	CH_3	**1p**	W110G	n.r.	-
			W110A	n.r.	-
			W110V	n.r.	-
			W110A/ I86A	46	98(*R*)

[a]Unless otherwise stated, reactions were performed using ketones **1k–p** (0.0191 mmol), TeSADH mutant (0.2 mg), $NADP^+$ (1.0 mg), Tris-HCl buffer solution (700 µL, 50 mM, pH 8.0), and 2-propanol (300 µL); for W110A/I86A TeSADH-catalyzed reduction reaction, $ZnCl_2$ (5 µL of 0.27 mM) and DTT (1.0 mg) were also added; for W110V TeSADH-catalyzed reduction reactions, **1k–p** (0.0038 mmol), Tris-HCl buffer solution (950 µL, 50 mM, pH 8.0) and 2-propanol (50 µL) were used. [b]Percent conversion was determined by GC. [c]The % *ee* of the corresponding acetate ester derivative of the alcohol produced was determined by GC using a chiral stationary phase. [n.r.: no reaction, n.d.: not determined]. [d]These results were obtained from Patel, J. M. *et al. Org. Biomol. Chem.* **2014**, *12* (31), 5905–5910 (reactions conducted in 5% of 2-propanol).

In an earlier report [47], the asymmetric reduction of 1-phenyl-2-propanone (**1k**) using W110G and W110A mutants of TeSADH produced the corresponding *S*-configured alcohol in excellent conversion and moderate *ee*, whereas the used of W110V resulted in significant enhancement in *ee* (> 99%) . Such an outcome was explained by the ability of this substrate to fit in dual modes within the large pocket of the active site of W110A and W110G mutants of TeSADH, whereas such dual fit is not allowed in W110V because of the smaller size of the large pocket. In the current report, W110A/I86A TeSADH-catalyzed reduction of **1k** resulted in a reverse stereochemical outcome, producing the corresponding *R*-configured alcohol in high conversion and good enantioselectivity (Table 10). The double mutant expands both pockets of TeSADH and

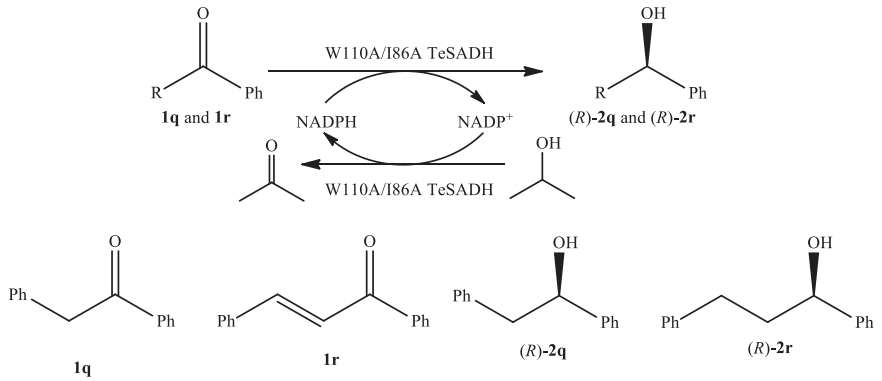
thus allowed the benzyl group to fit within the small pocket. This observation which indicated that the small pocket of TeSADH has higher affinity for hydrophobic moieties compered to its large pocket. The high binding affinity of the small pocket in the asymmetric reduction of aliphatic ketones using wild-type TbADH as well documented in the literature [7]. Asymmetric reduction of 1-(2'-chlorophenyl) propan-2-one (**1l**) by a s produced (*S*)-**2l** in high enantioselectivity, while using the double mutant resulted in the opposite enantiomer (*R*)-**2l** in high *ee* (99%). The presence of trifluoromethyl group (CF_3) in the meta position of 3-(trifluoromethyl)phenylacetone (**1m**) resulted in reduction to (*S*)-**2m** in high conversion and high enantioselectivity by all mutants. These observations indicated that the CF_3 on meta position does not allow substrate to fit within the small pocket of W110A/I86A TeSADH as was the case with **1k** and **1l** do. Likewise reduction of 1-phenyl-2-butanone (**1n**) predominantly produced the *S*-alcohol in high *ee*s when single mutants were used, whereas a moderate *ee* was observed by using the double mutant. The moderate enantioselectivity in the W110A/I86A TeSADH-catalyzed asymmetric reduction is due to the ability of the benzyl group to fit in the small pocket, giving a substantial amount of *R*-alcohol. Extending the alkyl moiety to propyl as in 1-phenyl-2-pentanone (**1o**) resulted in a reversed fit of this substrate in the active cite of W110A/I86A TeSADH producing (*R*)-**2o** in high *ee*. These findings revealed that indicated the propyl group enforces this substrate to fit in an orientation which facilitate the hydride delivery from the *Si* face of the ketone in *anti*-Prelog mode (i.e., benzyl moiety in the small pocket). This trend of reversed stereochemistry was also observed in the reduction of **1o**, using W110V TeSADH but with low conversion. Surprisingly, **1o** was not a substrate for W110G and W110A mutants of TeSADH.

W110A/I86A TeSADH-catalyzed reduction of acetophenone (**1p**), which is not a substrate for W110G, W110A, and W110V mutants of TeSADH, resulted in (*R*)-1-phenylethanol [(*R*)-**2p**] in high enantioselectivity and moderate conversion in (Table 10). The ketone **1p** is a substrate for I86A TeSADH as previously reported [45]. The stereochemical outcome with the double mutant in the asymmetric reduction of **1p** is consistent with the earlier report, wherein **1p** was reduced in *anti*-Prelog mode by using I86A TeSADH [45]. Compounds **1f-n** are substrates for W110 TeSADH mutants, but are not substrates for I86A TeSADH. Similarly **1p** is a substrate for I86A but not for W110 TeSADH mutants. Consequently, simultaneous mutations of W110A and I86A would generate a mutant that can reduce ketones, which are substrates for both individual mutants (i.e., W110A and I86A mutants of TeSADH). This striking finding prompted us to look for more interesting substrates like bulky-bulky ketones in order to expand substrate specificity of TeSADH.

Expanding the sizes of the two pockets of TeSADH in W110A/I86A TeSADH makes this mutant a potential catalyst to reduce bulky-bulky ketones such as 1,2-diphenylethanone (**1q**), 1,3-diphenyl-2-propen-1-one (**1r**), which are not substrates for W110A or I86A mutants of TeSADH. Reduction of **1q** using W110A/I86A TeSADH resulted in (*R*)-**2q** in high enantioselectivity and high conversion. The ability of this mutant to accommodate **1q** is attributed to the extension of both the small and large pockets of I86A/W110A TeSADH. Reduction of **1q** by W110A/I86A mutant followed Prelog rule with an inverted Cahn-Ingold-Prelog priority, which indicated that the benzyl and phenyl groups were accommodated by the large and small pockets, respectively. Reduction of such bulky-bulky substrate with high enantioselectivity is of great interest

due to the similar sizes of phenyl and benzyl moieties. Reduction of **1r** using W110A/I86A TeSADH produced (*R*)-1,3-diphenylpropan-1-ol [(*R*)-**2r**], which is result resulted from a Michael addition followed by reduction of the carbonyl function. W110A/I86A TeSADH-catalyzed reduction of **1r** followed Prelog rule, which indicated that due to the phenyl group fits within the small pocket of W110A/I86A TeSADH and thus resulted in a *Re* facial attack. The resultant enantiomers were found to be inseparable by both chiral GC or HPLC to determine the percent *ee* of the product alcohol. Activities of W110A/I86A TeSADH-catalyzed reduction of the bulky-bulky substrate such as **1q** and **1r** were clearly better than that of **1p**, which is a typical example of a small–bulky ketone. These observations are considered due to the proper fit of bulky-bulky substrates within the active site of W110A/I86A mutant that allowed for the maximum utilization of the active site.

Table 11: Asymmetric reduction of bulky-bulky ketones by W110A/I86A TeSADH.[a]

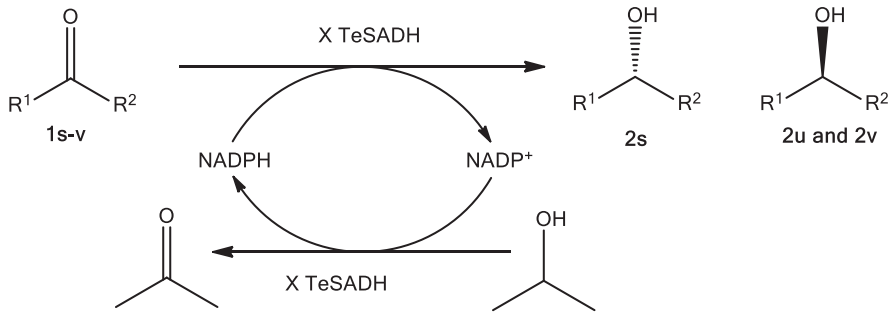


Substrate	Product	Conv. (%)[b]	*ee* (%)[c]
1q	(*R*)-**2q**	87	95
1r	(*R*)-**2r**	91	n.d.

[a]Unless otherwise stated, reactions were performed using ketone [**1q** or **1r** (0.0191 mmol)], W110A/I86A TeSADH (0.4 mg), $NADP^+$ (1.0 mg), Tris-HCl buffer solution (700 µL, 50 mM, pH 8.0), and 2-propanol (300 µL), $ZnCl_2$ (5 µL of 0.27 mM) and DTT (1.0 mg). [b]Percent conversion was determined by GC. [c]The % *ee* for (*R*)-**2q** was determined by HPLC hexane/methanol (v/v = 95: 5). n.d.: not determined.

An interesting characteristic of enzymes, besides their high enantioselectivity, is their high regioselectivity, a treat difficult to realize by This very important feature that is not easy to accomplish using organometallic or organic catalysts. Even though was observed in the asymmetric reduction of 1-phenyl-1,3-propanedione (**1s**), 1-phenyl-1,3-butanedione (**1u**), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (**1v**). Reduction of **1s** was not possible by using W110 single mutants of TeSADH. However, W110A/I86A TeSADH-catalyzed reduction of **1s** resulted in a regioselective reduction of the homobenzylic carbonyl to produce (*R*)-2-hydroxy-1-phenyl-1-propanone [(*R*)-**2s**] in excellent conversion (> 99%) and enantioselectivity (> 99%) (Table 12). The production of *R*-configured alcohol in asymmetric reduction of **1s** indicated that this substrate fits in an orientation that allowed *si*-facial attack of the carbonyl moiety (i.e., *anti*-Prelog mode). This stereochemical outcome was similar to that obtained by W110A/I86A TeSADH-catalyzed reduction of **1k** and **1l** (Table 10), which reflected the similarity among the three structures. Excellent performance was observed by all the mutants in the asymmetric reduction of **1u** in Prelog mode predominantly, yielding the (*S*)-3-hydroxy-1-phenyl-1-butanone [(*S*)-**2u**] leaving the benzylic carbonyl intact. Poor performances were observed in the asymmetric reduction of **1v** using W110A, W110G and W110A/I86A mutants of TeSADH. Surprisingly, W110V TeSADH-catalyzed reduction of **1v** that resulted in (*R*)-4,4,4-trifluoro-3-hydroxy-1-phenyl-1-butanone [(*R*)-**2v**] in excellent conversion (> 99%), enantioselectivity [> 99%] and regionselectivity leaving the benzylic carbonyl intact. The (*R*)-configured alcohol produced in the asymmetric reduction of **1v** is a result of an inverse Cahn-Ingold-Prelog priority (i.e., the reaction follows Prelog rule).

Table 12: Regio- and enantioselective reduction of phenyl-ring-containing diketones by different mutants of TeSADH.[a]



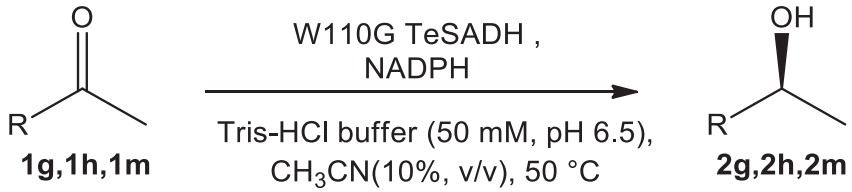
R^1	R^2	Substrate	X	Product	Conv. (%)[b]	*ee*(%)[c]
PhCO	CH_3	**1s**	W110G	(*R*)-**2s**	n.r.	-
			W110A		n.r.	-
			W110V		n.r.	-
			W110A/I86A		> 99	> 99
$PhCOCH_2$	CH_3	**1u**	W110G	(*S*)-**2u**	92	> 99
			W110A[e]		99	> 99
			W110V		89	> 99
			W110A/I86A		92	92
$PhCOCH_2$	CF_3	**1v**	W110G	(*R*)-**2v**	3	n.d.
			W110A		8	n.d.
			W110V		> 99	> 99[d]
			W110A/I86A		2	n.d.

[a]Unless otherwise stated, reactions were performed using ketone [**1s**–**v** (0.0191 mmol)], TeSADH mutant (0.2 mg), $NADP^+$ (1.0 mg), Tris-HCl buffer solution (700 µL, 50 mM, pH 8.0), and 2-propanol (300 µL); for W110A/I86A TeSADH-catalyzed reduction reactions, $ZnCl_2$ (5 µL of 0.27 mM) and DTT (1.0 mg) were also added; for W110V TeSADH-catalyzed reduction reactions, [**1s**–**v** (0.0038 mmol)], Tris-HCl buffer solution (950 µL, 50 mM, pH 8.0), and 2-propanol (50 µL) were used. [b]Percent conversion was determined by GC. [c]The % *ee* of the corresponding acetate ester derivative of the alcohol produced was determined by GC using a chiral stationary phase. [d]The % *ee* of (*R*)-**2v** was determined by HPLC using hexane/2-propanol (95:5, v/v). [e]Results obtained from Musa, M. M. *et al. J. Org. Chem.* **2007**, *72* (2), 30–34.

The effect of a substituent on the phenyl ring of ketones was investigated by measuring the Michaelis-Menten constant (K_m) and maximum rate (V_{max}) for W110G TeSADH-catalyzed reduction reactions of **1g**, **1h**, and **1m** (Table 13). The highest K_m value was for **1g** was observed suggesting a low binding affinity of this substrate within the active site of W110G TeSADH, which is consistent with the observed low

enantioselectivities observed in the asymmetric reduction of this substrate compared to its obtained for **1h** and **1m** (Table 10). The low K_m value obtained for **1h** could be attributed to the high binding affinity of the hydroxyl group with the active site of W110G TeSADH. The least K_m was obtained with **1m** which is also consistent with the observed high enantioselectivity (96%) in the W110G TeSADH-catalyzed reduction of this substrate. High binding affinity of the substrate at the active site leads to a more rigid substrate orientation reducing selectivity mistakes, and hence improved enantioselectivity.

Table 13: Kinetic parameters for the asymmetric reduction of ketones using W110G TeSADH.[a]



Substrate	K_m (mM)	V_{max} (mM.min^{-1})
1g	2.58418 ± 0.00992	0.08775 ± 0.00225
1h	0.6993 ± 0.0462	0.04779 ± 0.00316
1m	0.33455 ± 0.0160	0.0743 ± 0.00356

[a]Reactions were performed at 50 °C by using ketone [**1g**, **1h** or **1m** (0.005-15 mM)], W110G TeSADH mutant (0.002 mg), NADPH (0.25 mM), and acetonitrile (10%, v/v) in Tris-HCl buffer solution (50 mM, pH 6.5). R as defined in Table 9 and table 10.

Chapter 5

CONCLUSION

In conclusion, we have demonstrated that the double mutation of TeSADH at W110 and I86 sites expand substrate scope for W110A/I86A TeSADH. Subsequently, it reduces ketones that are substrates for W110 mutants of TeSADH and I86A TeSADH with comparable activities and enantioselectivities compered to reduction by the single mutants. The expanded pockets of this double mutant also accommodates ketones bearing bulky groups on both sides of the carbonyl like **1q** and **1r** with high activities and enantioselectivities. The asymmetric reduction of phenyl-ring-containing ketones was undertaking by using W110G, W110A, and W110V mutants of TeSADH and compared it with those obtained by W110A/I86A TeSADH. A reverse stereochemical outcome was observed the position of the subsistent on the phenyl ring of the 2-teralone was changed. Kinetic results indicated that the K_m does not only depend on the enzyme mutation, but also on the position of the substituent on the aromatic ring of the 2-tetralone. In addition, a reverse enantiopreference for few substrates was realized when W110A/I86A TeSADH was used. We were also succeeded in conducting regionselective reductions of diketones using both the double or single mutants of TeSADH, a feature that is tough to accomplish by non-enzymatic approaches. The ability of W110A/I86A TeSADH to accommodate a wider scope of substrates than those accommodated by wild-type TeSADH and other previously reported single mutants will be of great interest to organic chemists.

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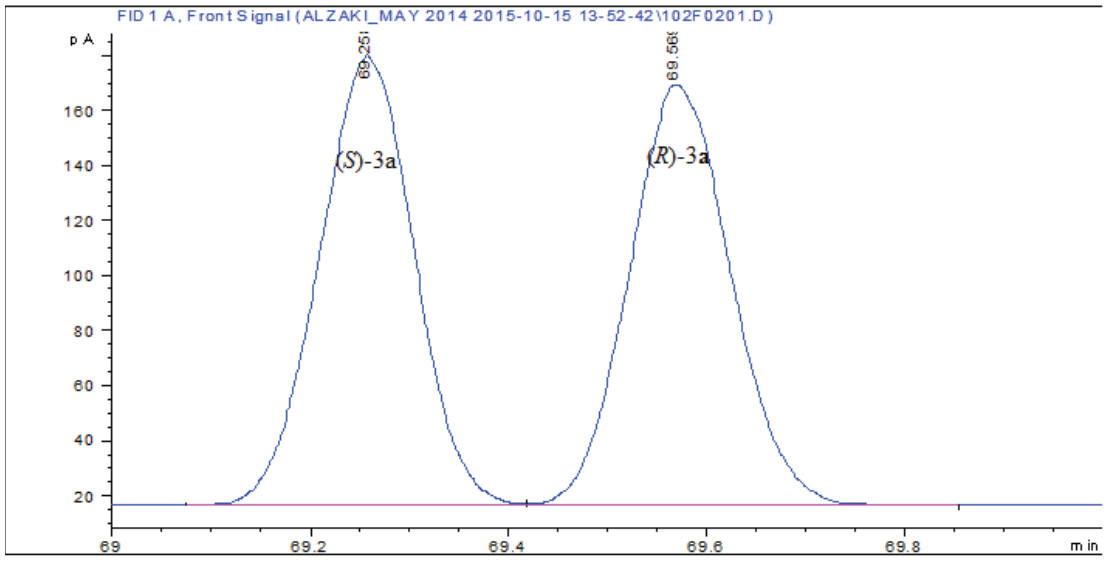
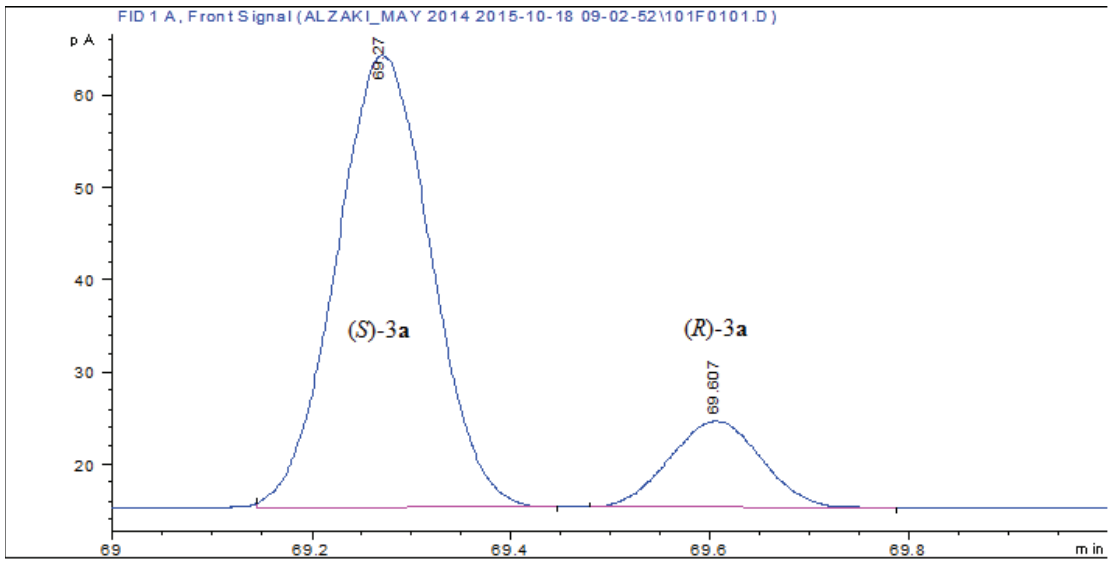
Appendix

1. GC Chromatograms

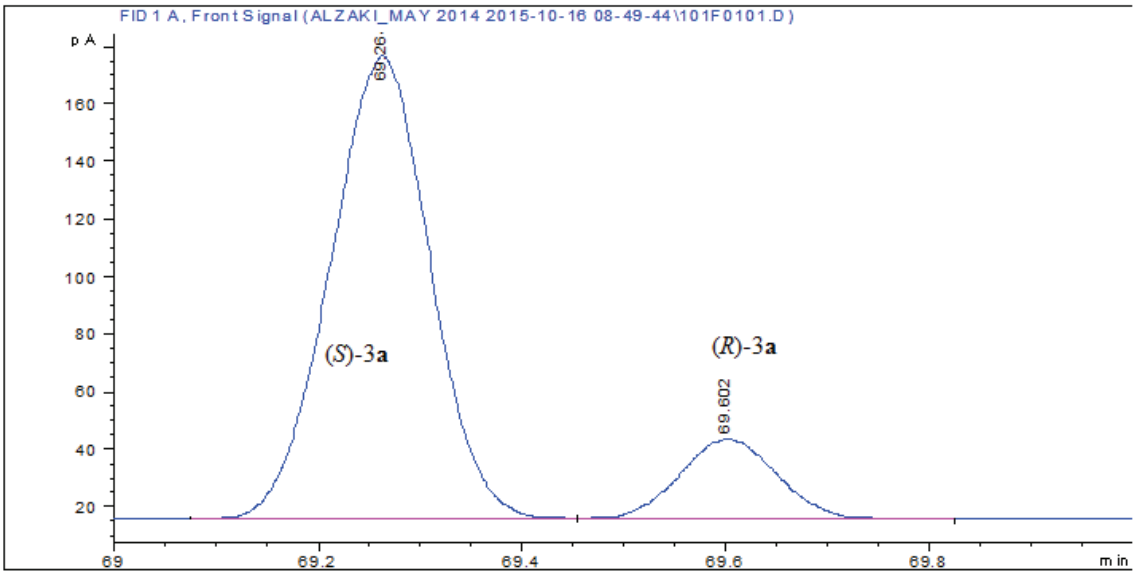
a)



b)



c)



d)

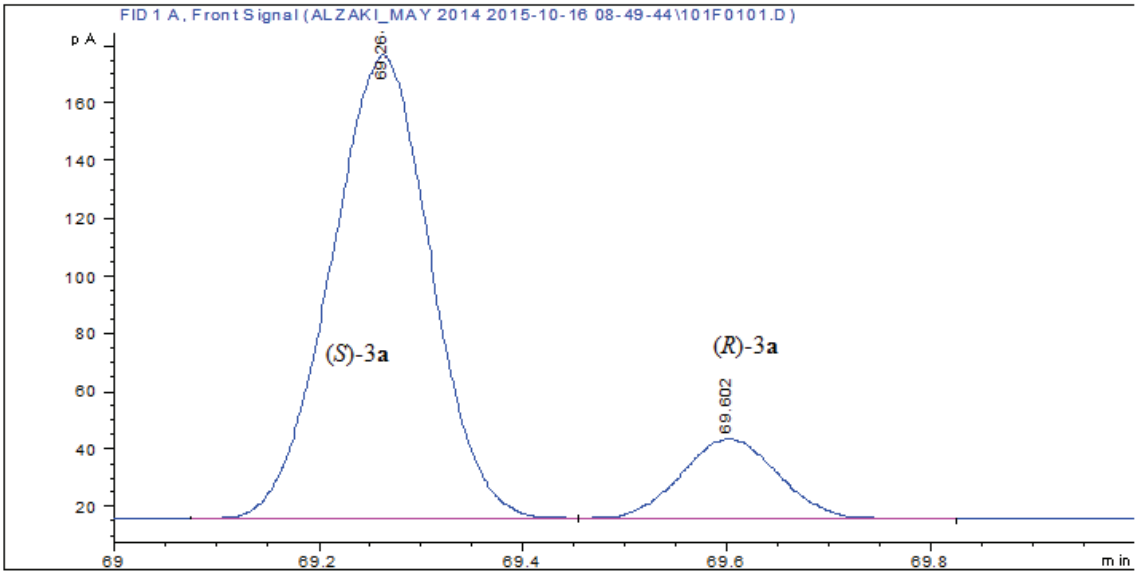
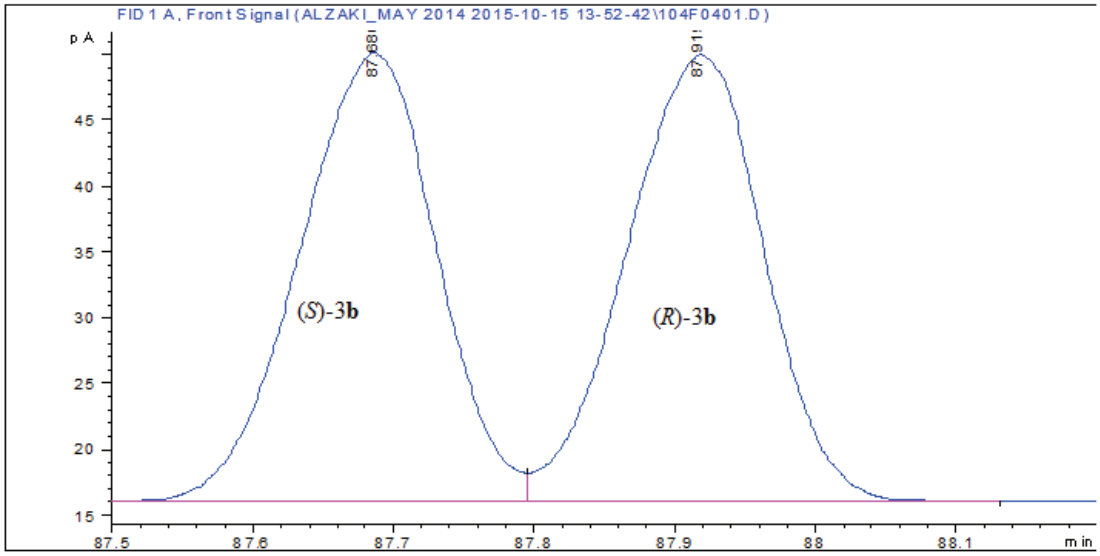
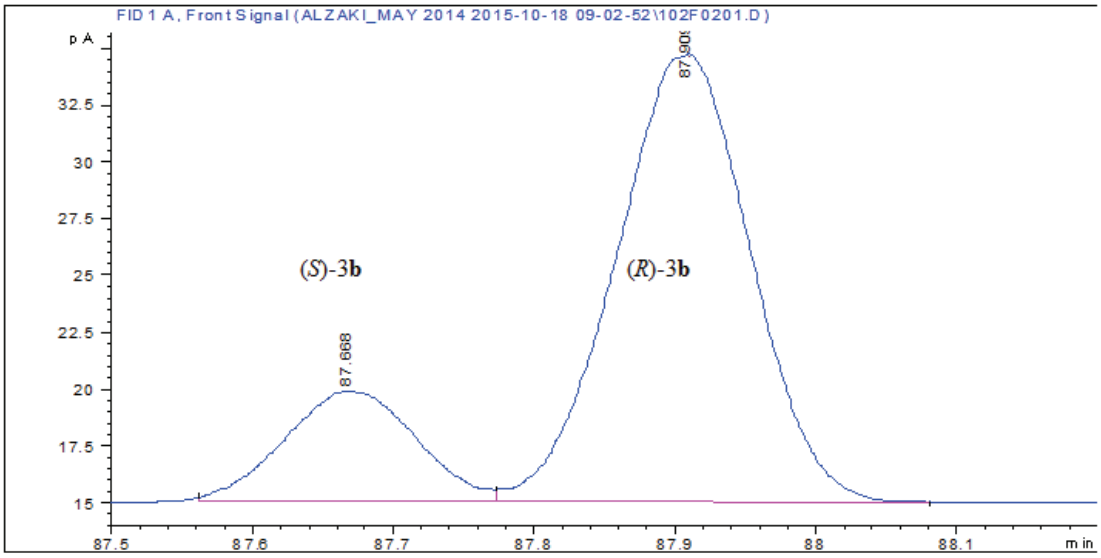


Figure A15. GC Chromatogram of: a) acetate derivative of *rac*-**2a** (made by reduction of **1a** with $NaBH_4$), b) acetate derivative of (*S*)-**2a** prepared by W110G TeSADH, c) acetate derivative of (*S*)-**2a** prepared by W110A TeSADH, d) acetate derivative of (*S*)-**2a** prepared by W110A/I86A TeSADH.

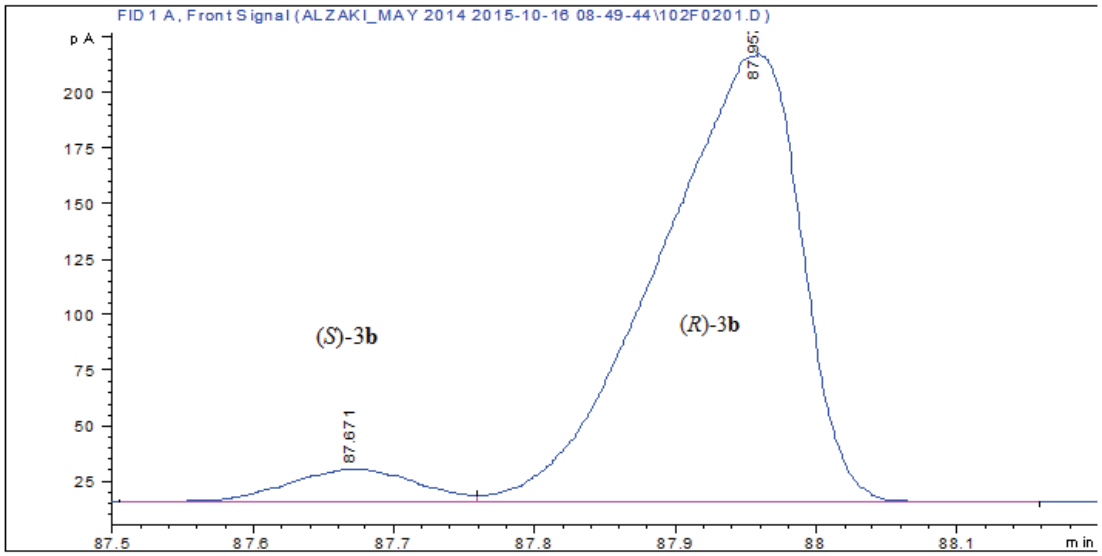
a)


FID 1 A, Front Signal (ALZAKI_MAY 2014 2015-10-15 13-52-42\104F0401.D)
pA
45
40
35
30
25
20
15
87.668
87.911
(S)-3b
(R)-3b
87.5
87.6
87.7
87.8
87.9
88
88.1
min

b)


FID 1 A, Front Signal (ALZAKI_MAY 2014 2015-10-18 09-02-52\102F0201.D)
pA
32.5
30
27.5
25
22.5
20
17.5
15
87.668
87.906
(S)-3b
(R)-3b
87.5
87.6
87.7
87.8
87.9
88
88.1
min

c)



d)

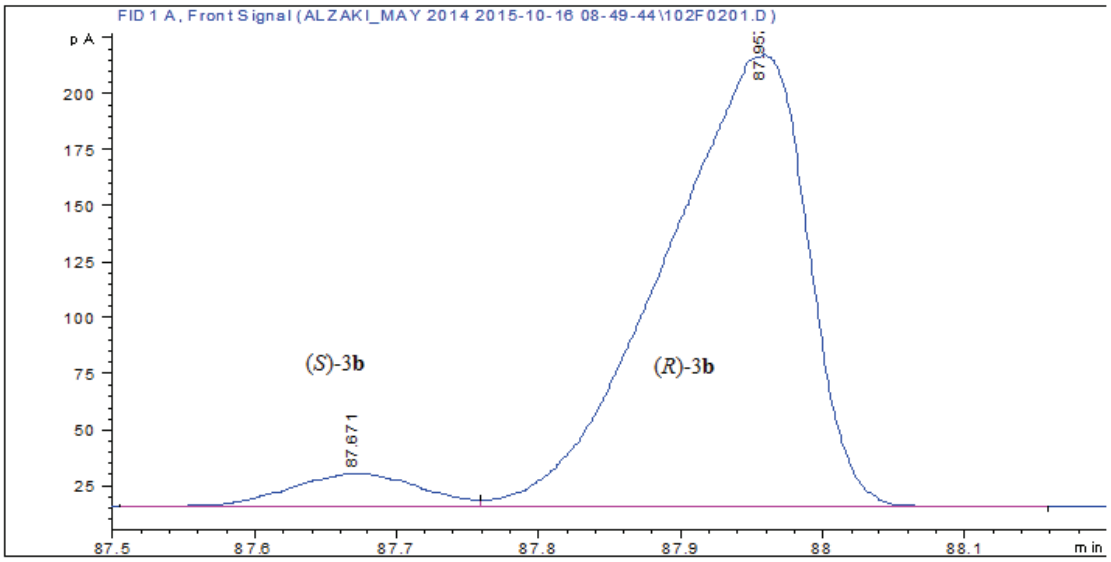
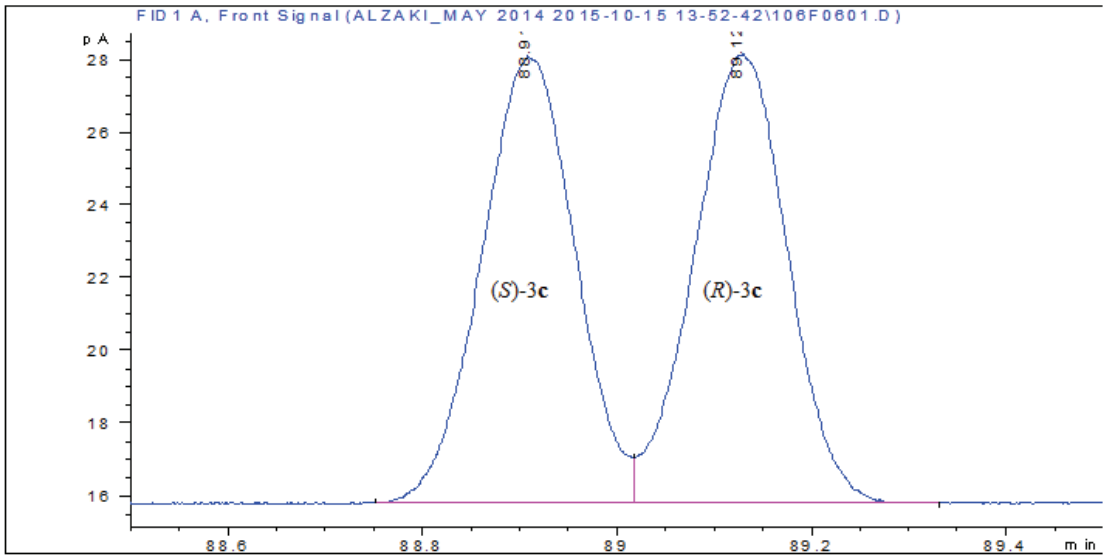
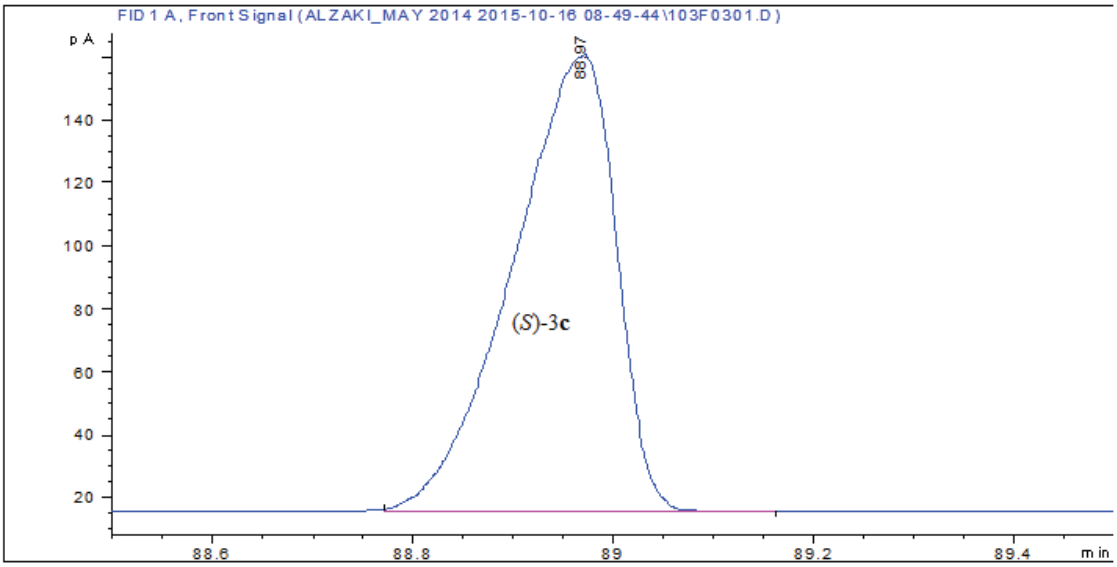


Figure A16. GC Chromatogram of: a) acetate derivative of *rac*-**2b** (made by reduction of **1b** with $NaBH_4$), b) acetate derivative of (*S*)-**2b** prepared by W110G TeSADH, c) acetate derivative of (*S*)-**2b** prepared by W110A TeSADH, d) acetate derivative of (*S*)-**2b** prepared by W110A/I86A TeSADH.

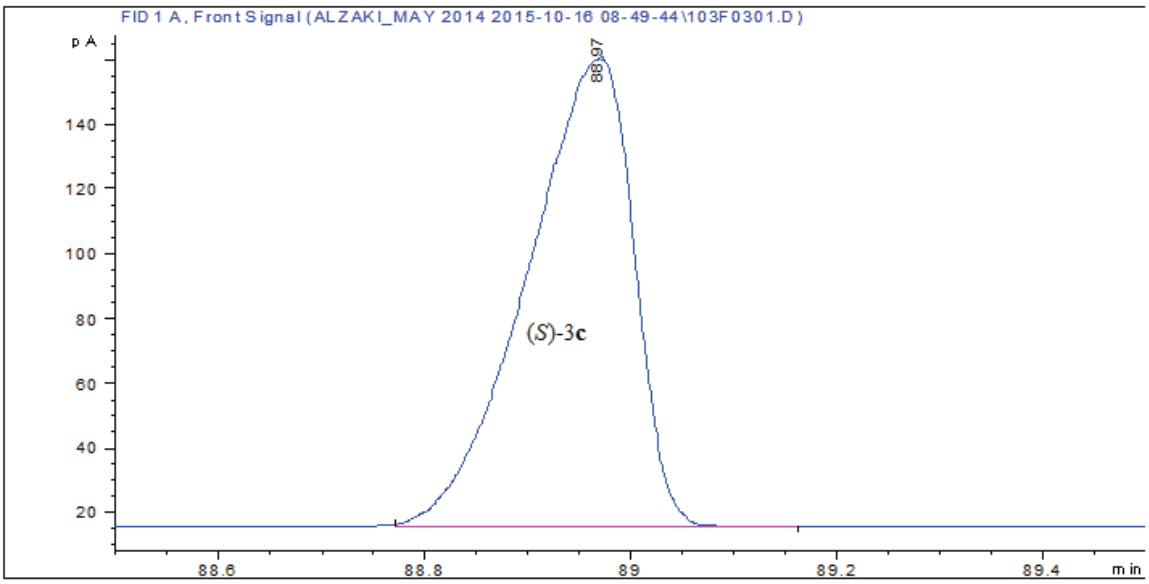
a)


FID 1 A, Front Signal (ALZAKI_MAY 2014 2015-10-15 13-52-42\106F0601.D)
pA
28
26
24
22
20
18
16
88.9
89.1
(S)-3c
(R)-3c
88.6
88.8
89
89.2
89.4
min

b)


FID 1 A, Front Signal (ALZAKI_MAY 2014 2015-10-16 08-49-44\103F0301.D)
pA
140
120
100
80
60
40
20
88.97
(S)-3c
88.6
88.8
89
89.2
89.4
min

c)



d)

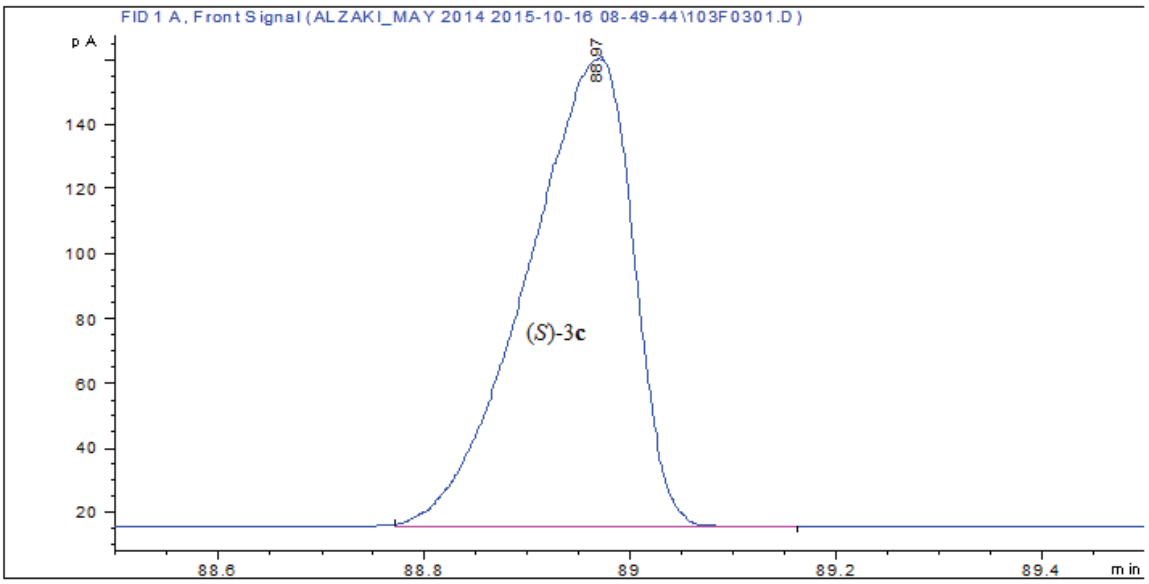
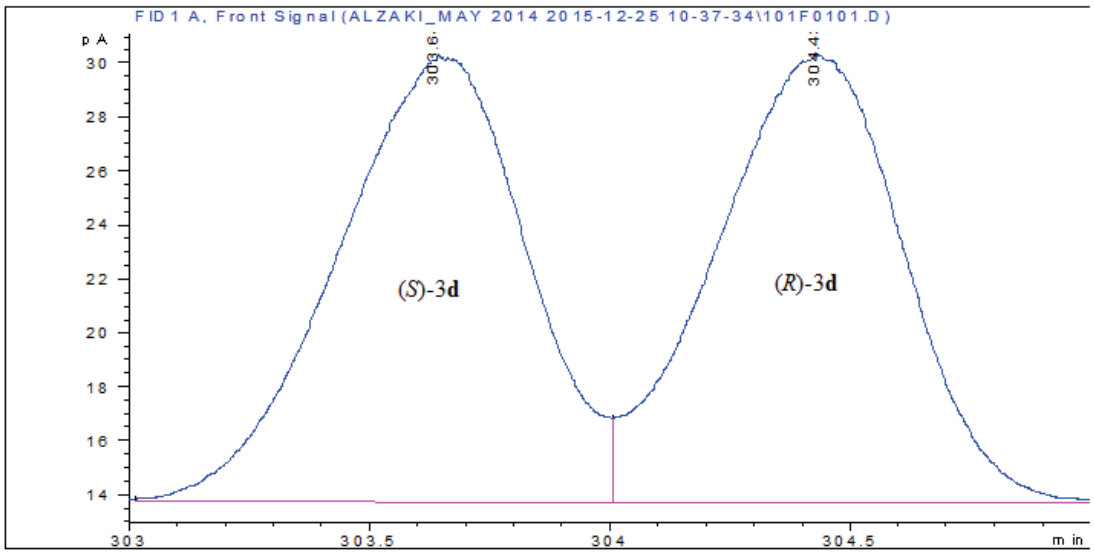
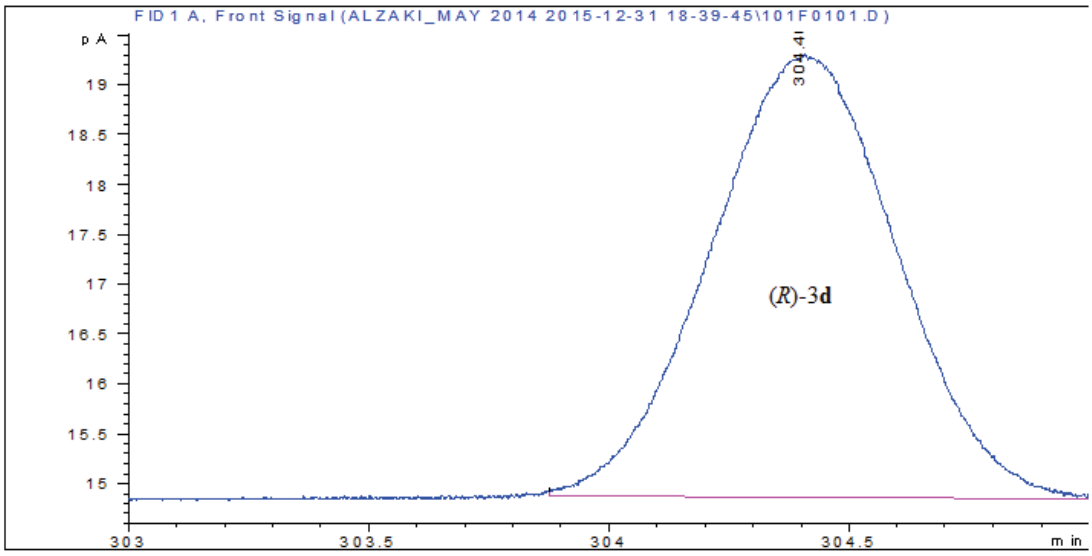


Figure A17. GC Chromatogram of: a) acetate derivative of *rac*-**2c** (made by reduction of **1c** with $NaBH_4$), b) acetate derivative of (*S*)-**2c** prepared by W110G TeSADH, c) acetate derivative of (*S*)-**2c** prepared by W110A TeSADH, d) acetate derivative of (*S*)-**2c** prepared by W110A/I86A TeSADH.

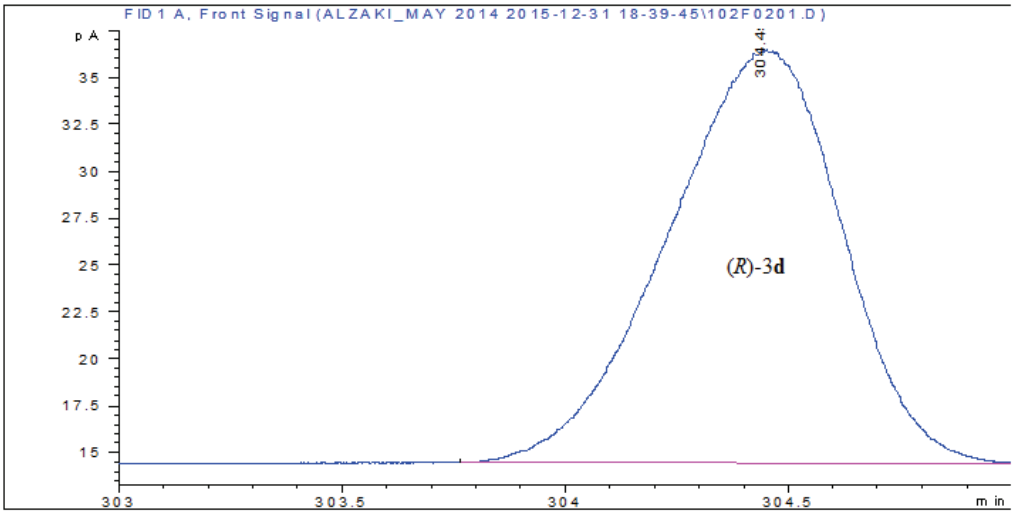
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2015-12-25 10-37-34\101F0101.D)
pA
303.6
304.4
(S)-3d
(R)-3d
min

b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2015-12-31 18-39-45\101F0101.D)
pA
304.4
(R)-3d
min

c)



d)

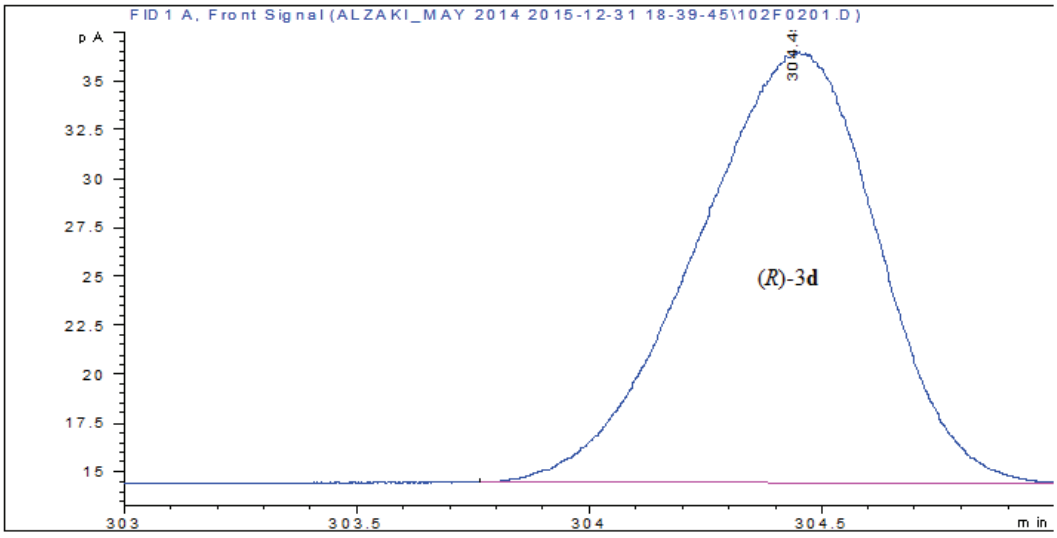
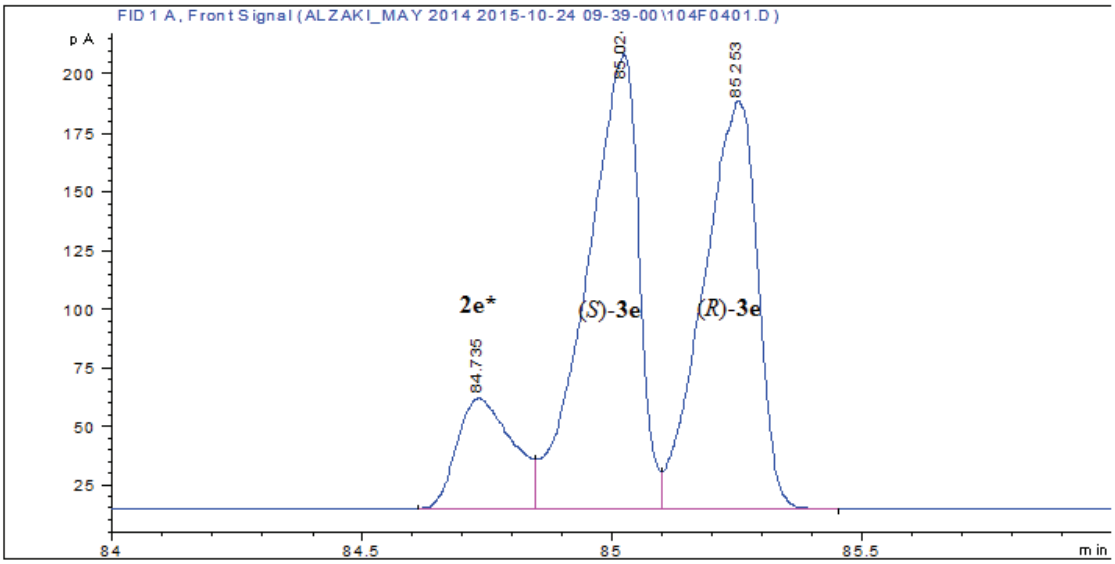


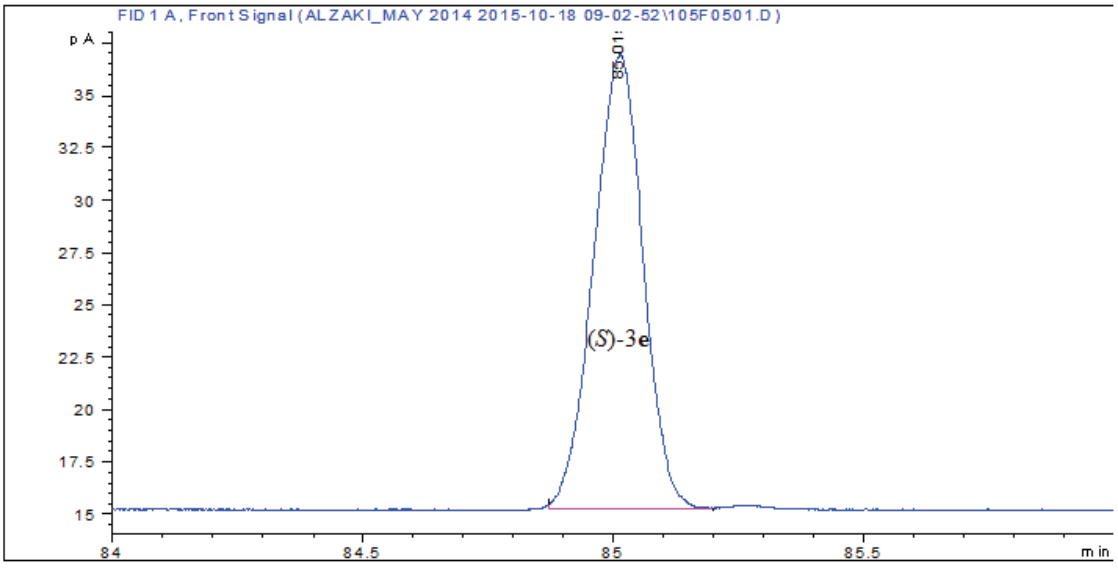
Figure A18. GC Chromatogram of: a) acetate derivative of *rac*-**2d** (made by reduction of **1d** with $NaBH_4$), b) acetate derivative of (*S*)-**2d** prepared by W110G TeSADH, c) acetate derivative of (*S*)-**2d** prepared by W110A TeSADH, d) acetate derivative of (*S*)-**2d** prepared by W110A/I86A TeSADH.

a)

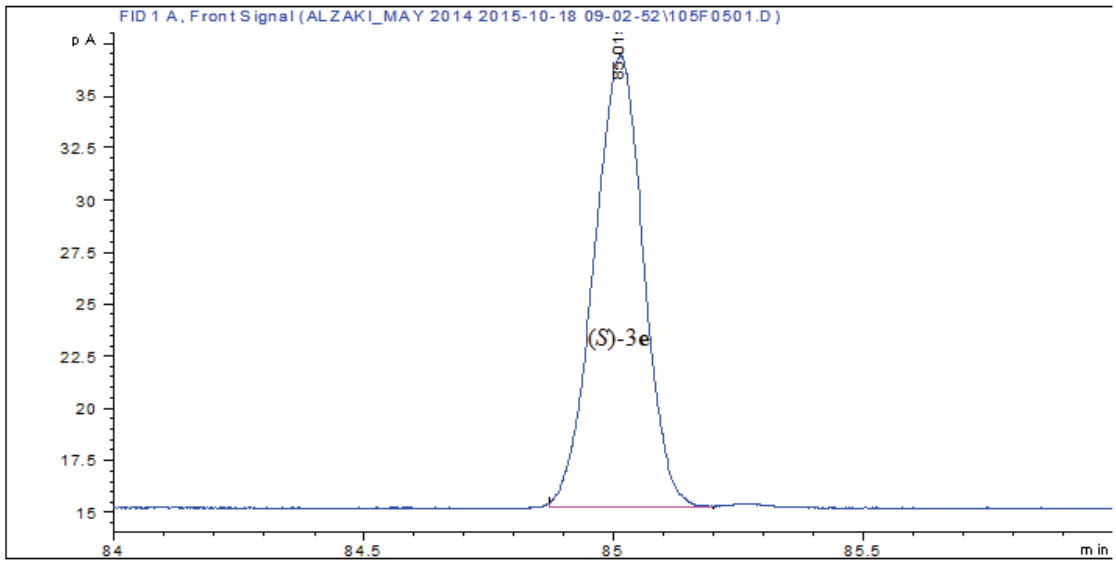


* Alcohol product due to incomplete derivatization.

b)



c)



d)

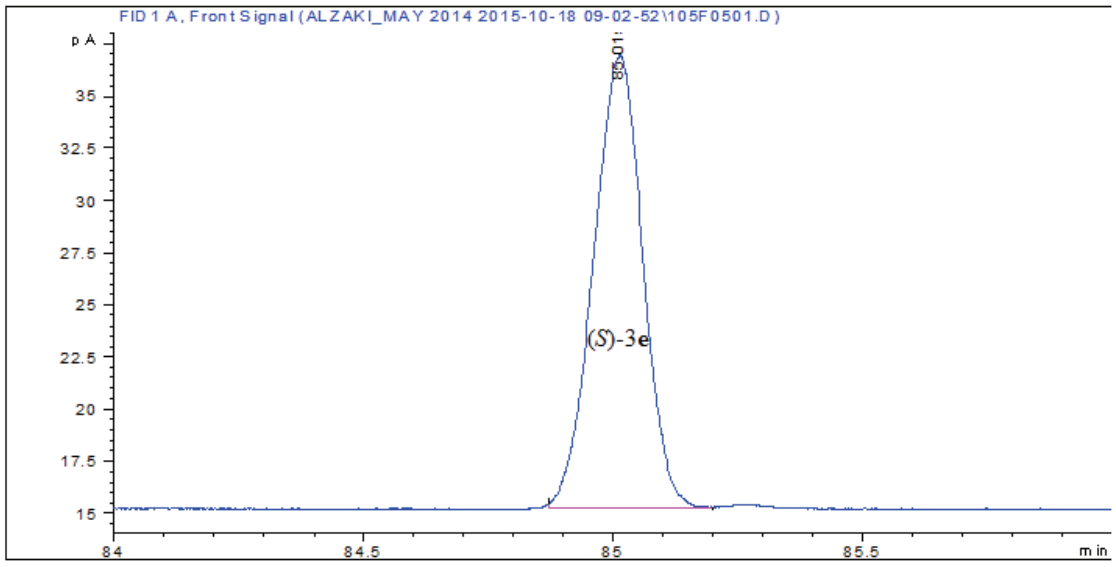
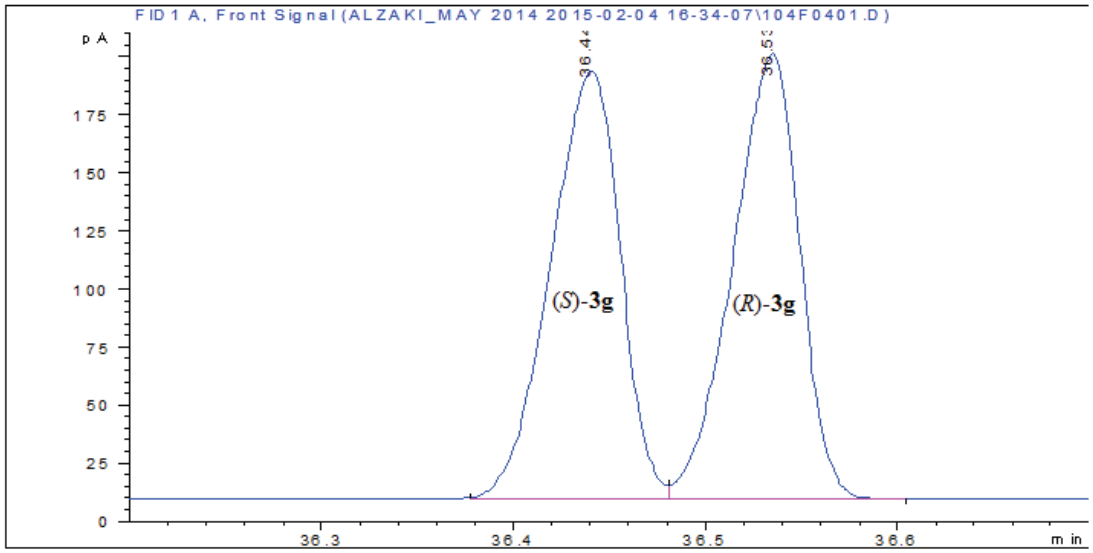
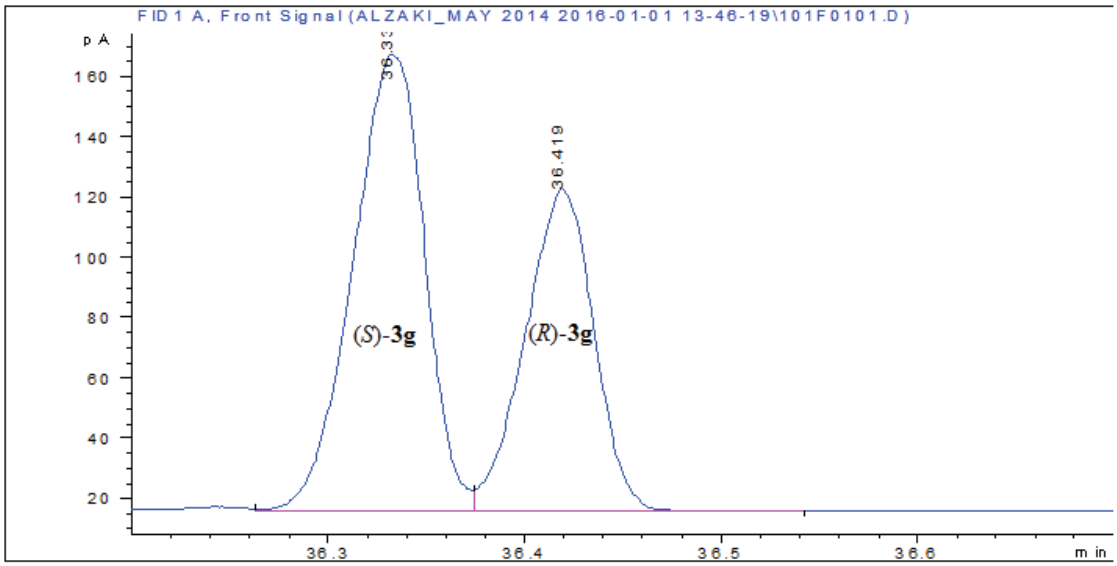


Figure A19. GC Chromatogram of: a) acetate derivative of *rac*-**2e** (made by reduction of **1e** with $NaBH_4$), b) acetate derivative of (*S*)-**2e** prepared by W110G TeSADH, c) acetate derivative of (*S*)-**2e** prepared by W110A TeSADH, d) acetate derivative of (*S*)-**2e** prepared by W110A/I86A TeSADH.

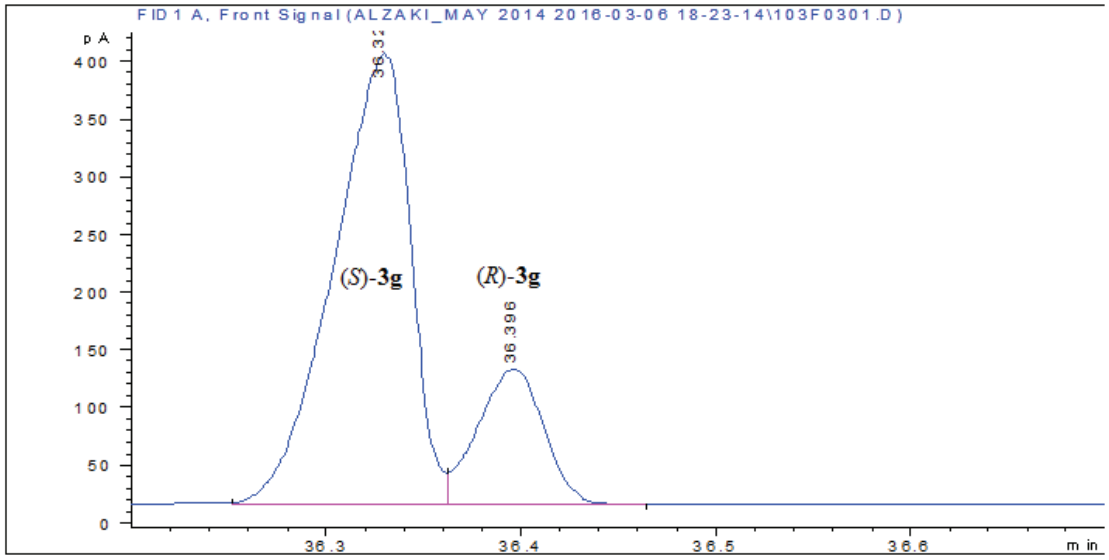
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2015-02-04 16-34-07\104F0401.D)
pA
175
150
125
100
75
50
25
0
36.4
36.5
(S)-3g
(R)-3g
36.3
36.4
36.5
36.6
min

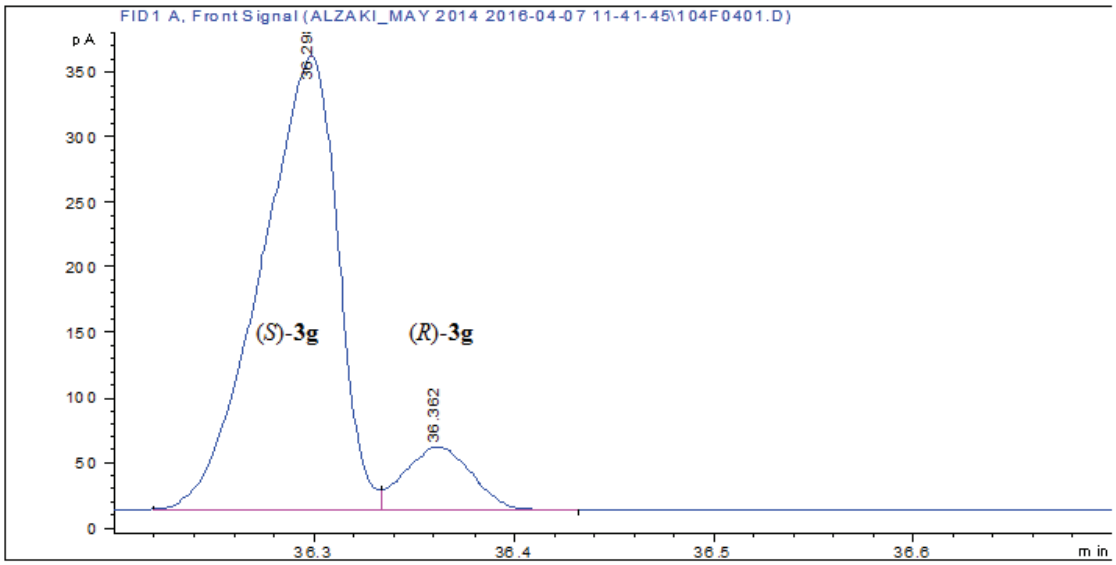
b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-01-01 13-46-19\101F0101.D)
pA
160
140
120
100
80
60
40
20
36.419
(S)-3g
(R)-3g
36.3
36.4
36.5
36.6
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-06 18-23-14\103F0301.D)
pA
400
350
300
250
200
150
100
50
0
36.31
(S)-3g
(R)-3g
36.396
36.3
36.4
36.5
36.6
min

d)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-07 11-41-45\104F0401.D)
pA
350
300
250
200
150
100
50
0
36.29
(S)-3g
(R)-3g
36.362
36.3
36.4
36.5
36.6
min

e)

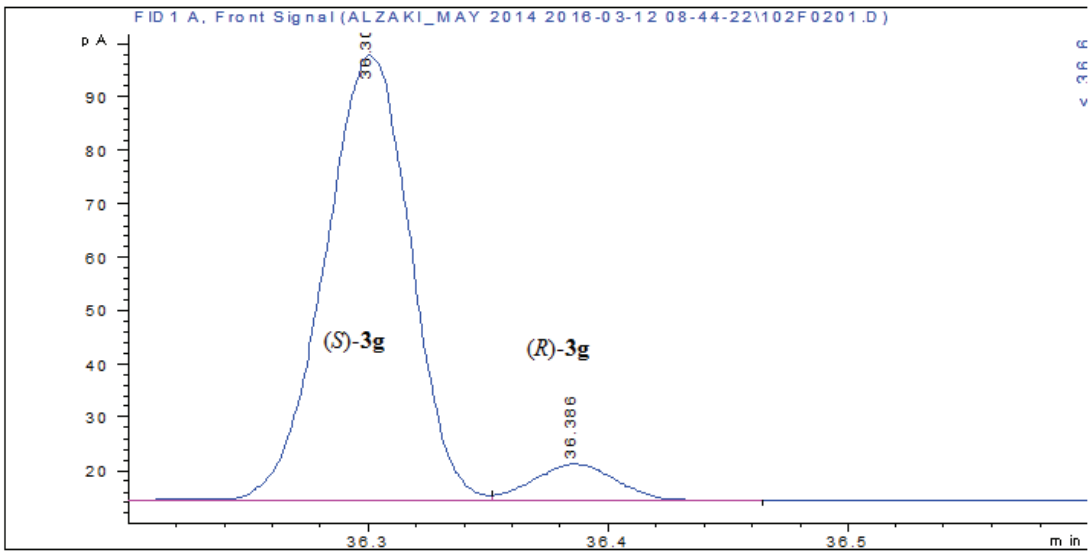
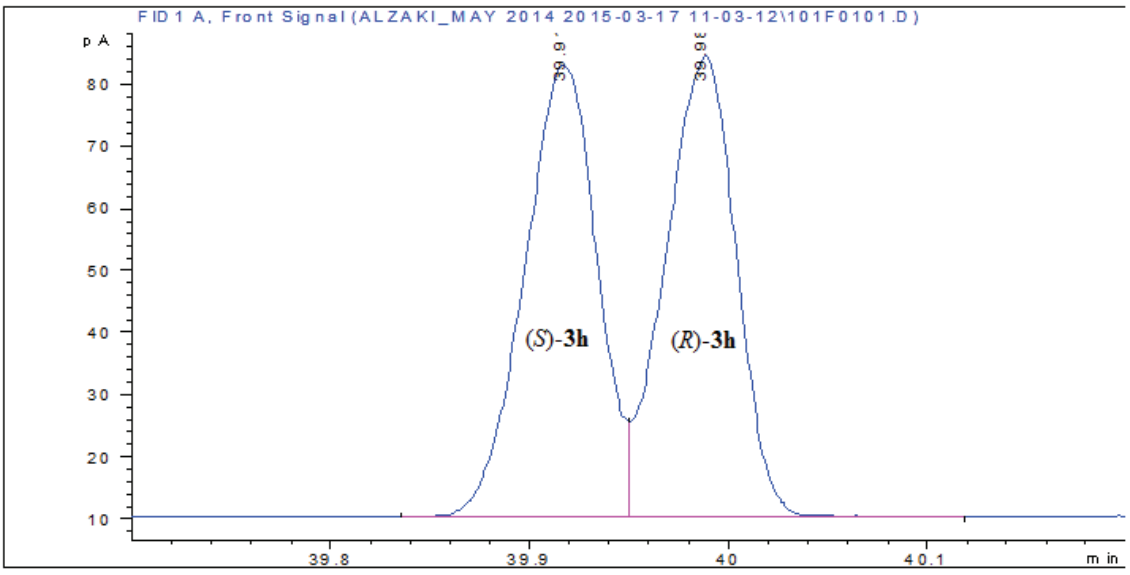
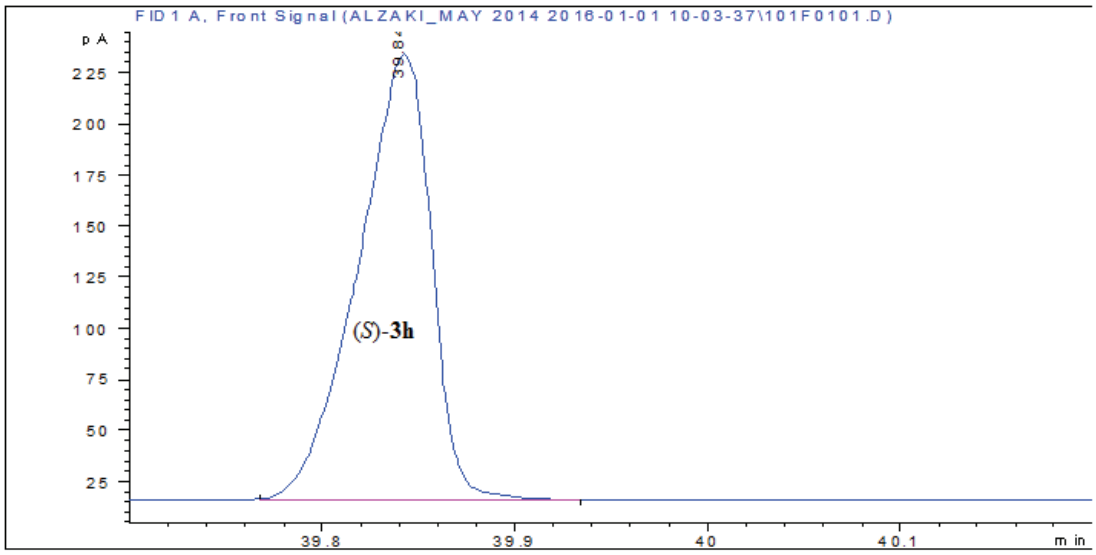


Figure A6. GC Chromatogram of: a) acetate derivative of *rac*-**2g** (made by reduction of **1g** with $NaBH_4$), b) acetate derivative of (*S*)-**2g** with W110G TeSADH, c) acetate derivative of (*S*)-**2g** with W110A TeSADH, d) acetate derivative of (*S*)-**2g** with W110V TeSADH, e) acetate derivative of (*S*)-**2g** with W110A/I86A TeSADH.

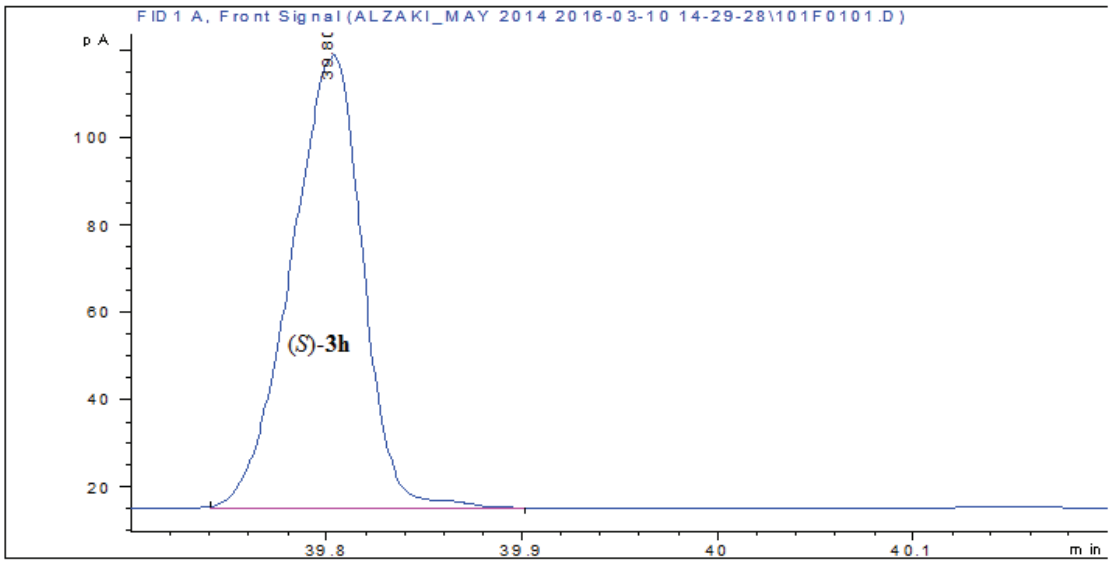
a)



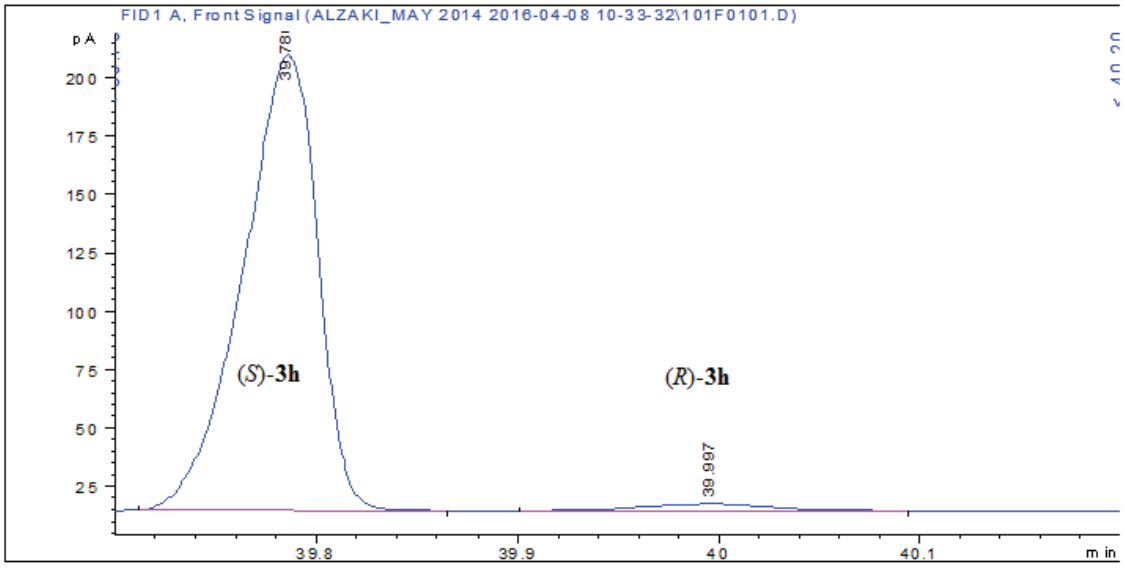
b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-01-01 10-03-37\101F0101.D)
pA
225
200
175
150
125
100
75
50
25
39.84
(S)-3h
39.8
39.9
40
40.1
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-10 14-29-28\101F0101.D)
pA
100
80
60
40
20
39.81
(S)-3h
39.8
39.9
40
40.1
min

d)



e)

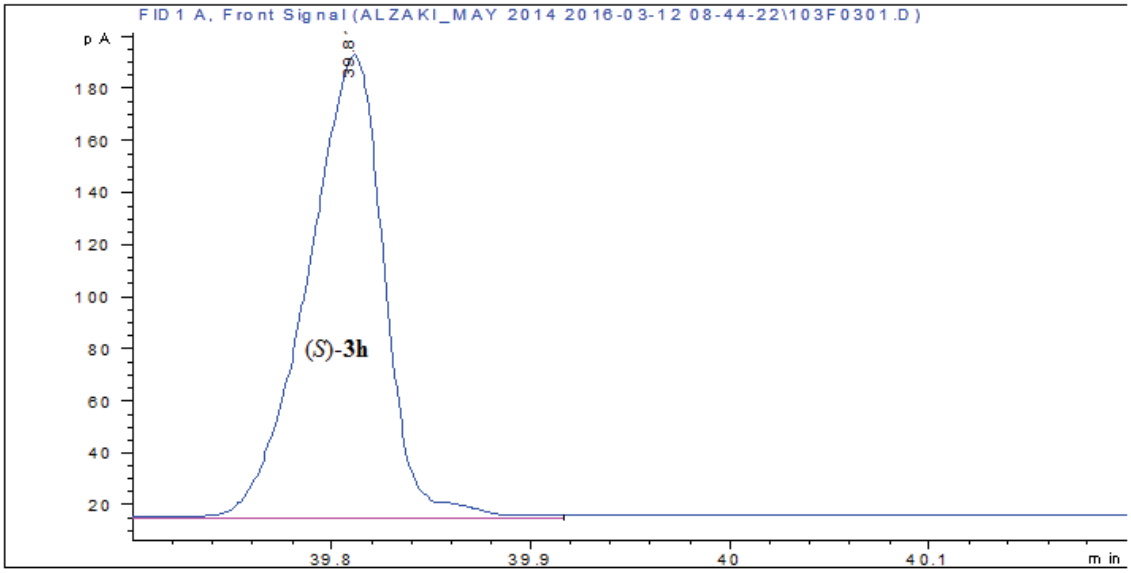
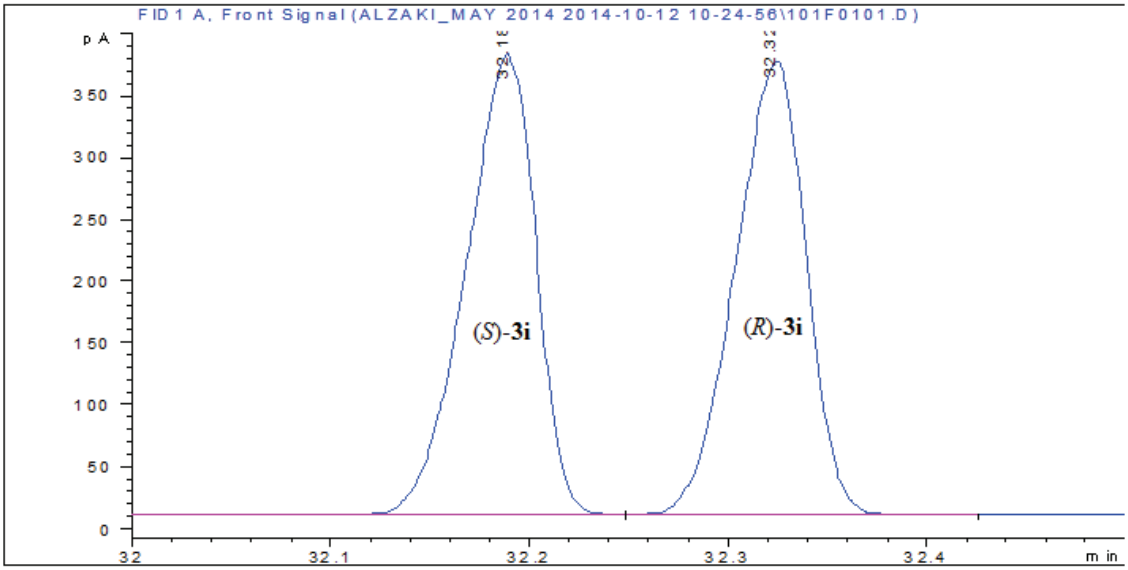
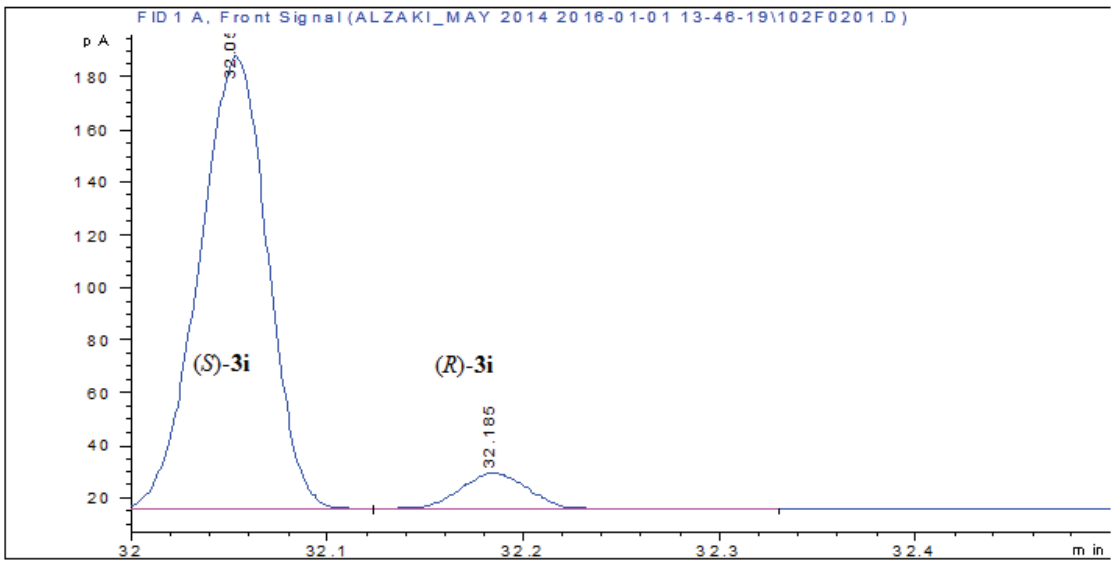


Figure A7. GC Chromatogram of: a) acetate derivative of *rac*-**2h** (made by reduction of **1h** with $NaBH_4$), b) acetate derivative of (*S*)-**2h** W110G TeSADH, c) acetate derivative of (*S*)-**2h** W110A TeSADH, d) acetate derivative of (*S*)-**2h** with W110V TeSADH, e) acetate derivative of (*S*)-**2h** W110A/I86A TeSADH.

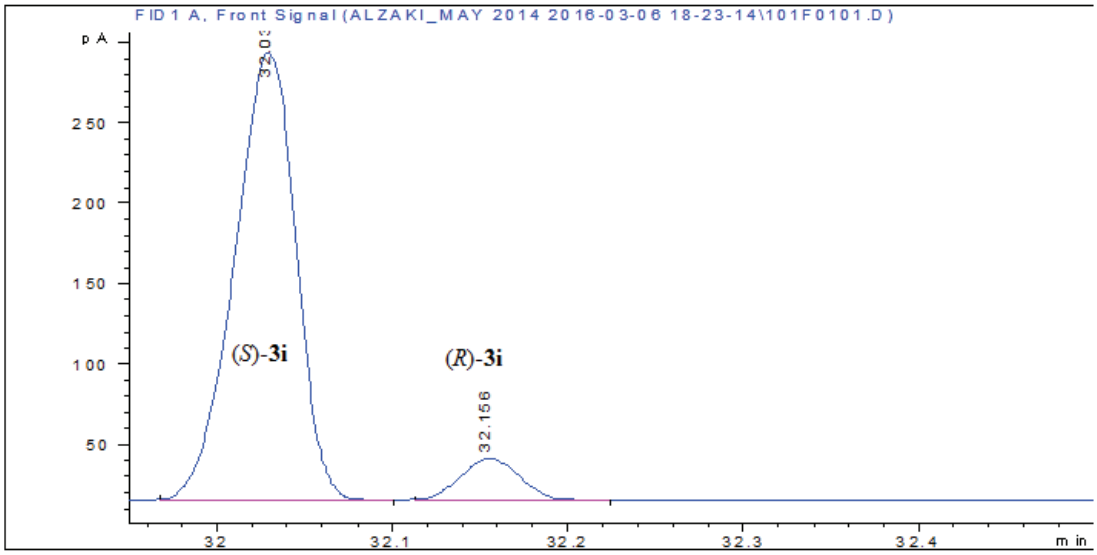
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2014-10-12 10-24-56\101F0101.D)
pA
350
300
250
200
150
100
50
0
(S)-3i
(R)-3i
32
32.1
32.2
32.3
32.4
min

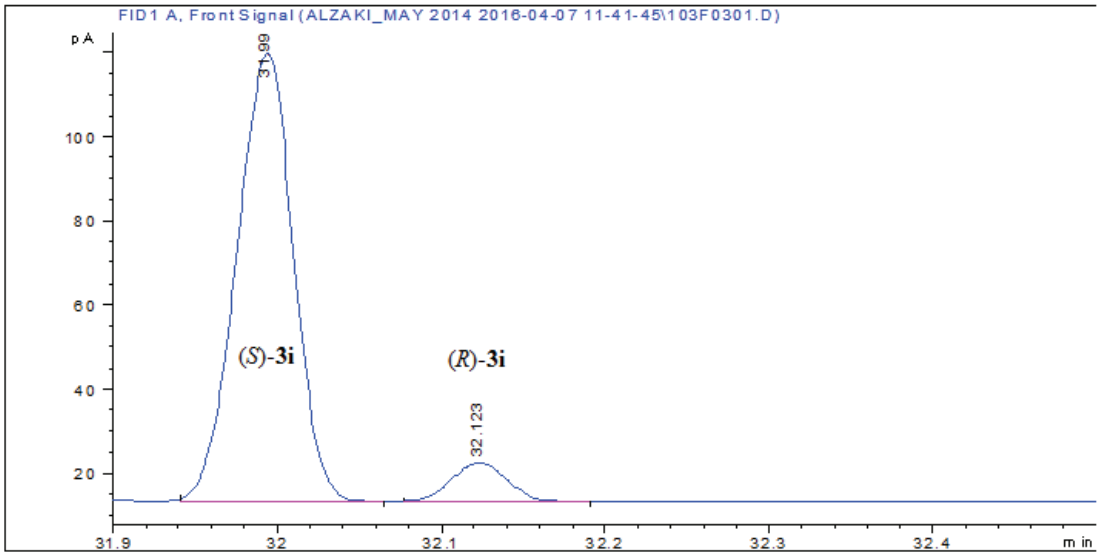
b)

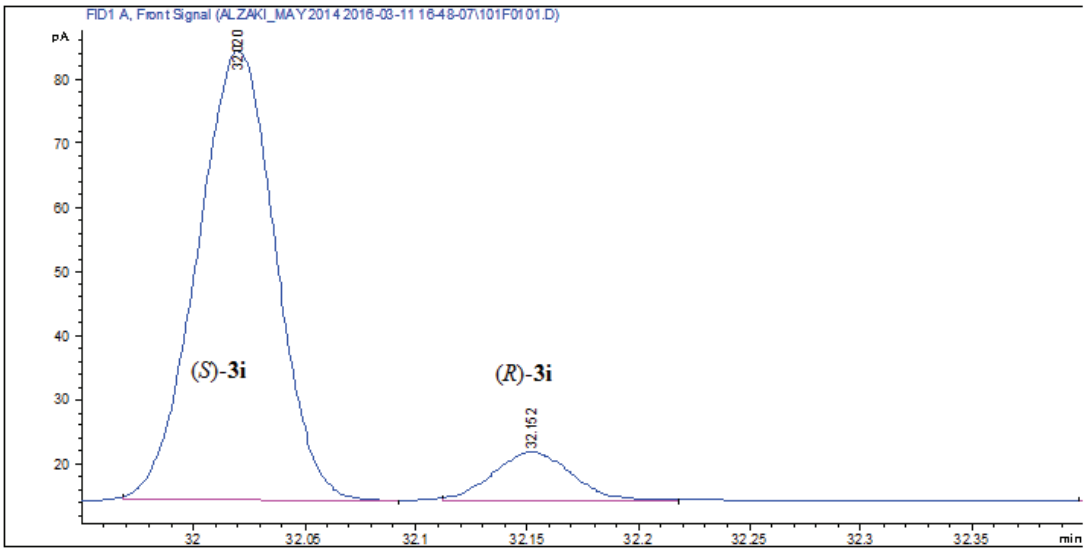

FID1 A, Front Signal (ALZAKI_MAY 2014 2016-01-01 13-46-19\102F0201.D)
pA
180
160
140
120
100
80
60
40
20
(S)-3i
(R)-3i
32.185
32
32.1
32.2
32.3
32.4
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-06 18-23-14\101F0101.D)
pA
250
200
150
100
50
32.05
(S)-3i
(R)-3i
32.156
32
32.1
32.2
32.3
32.4
min

d)

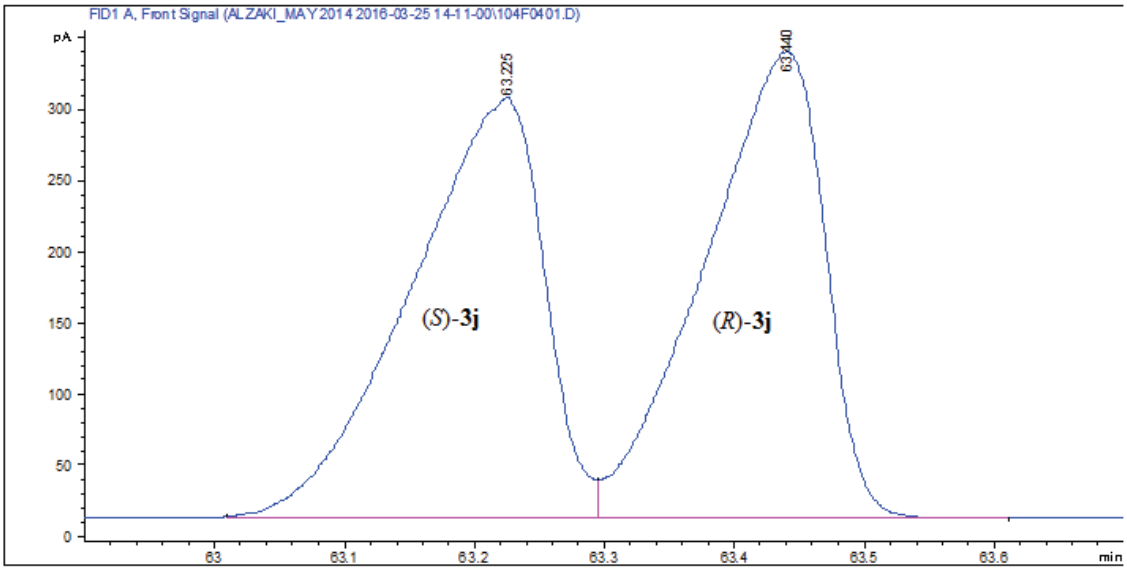

FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-07 11-41-45\103F0301.D)
pA
100
80
60
40
20
31.99
(S)-3i
(R)-3i
32.123
31.9
32
32.1
32.2
32.3
32.4
min



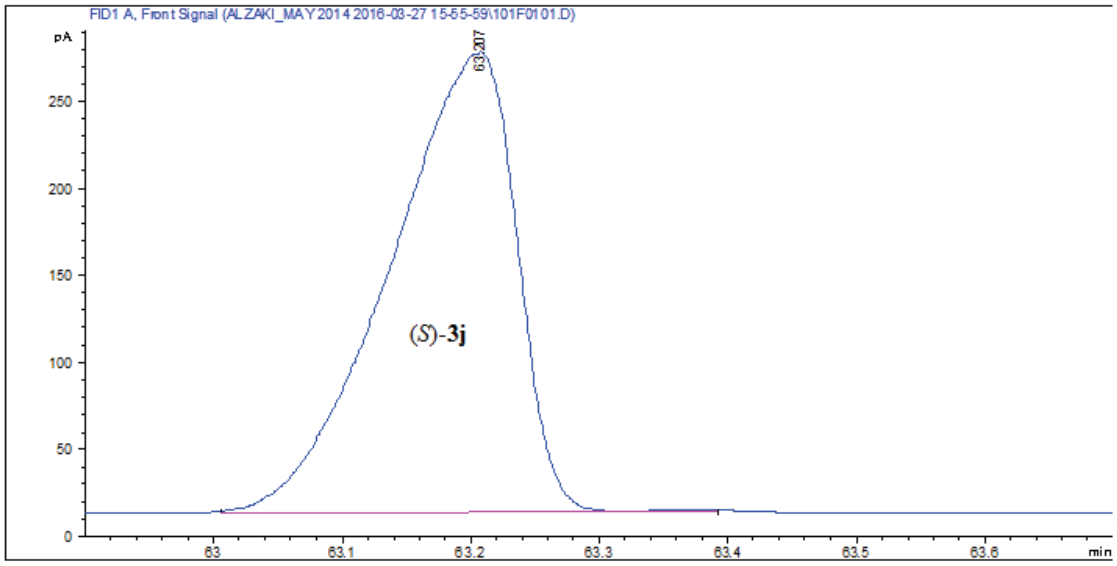
e)

Figure A8. GC Chromatogram of: a) acetate derivative of *rac*-**2i** (made by reduction of **1i** with NaBH4), b) acetate derivative of (*S*)-**2i** W110G TeSADH, c) acetate derivative of (*S*)-**2i** W110A TeSADH, d) acetate derivative of (*S*)-**2i** with W110V TeSADH, e) acetate derivative of (*S*)-**2i** W110A/I86A TeSADH.

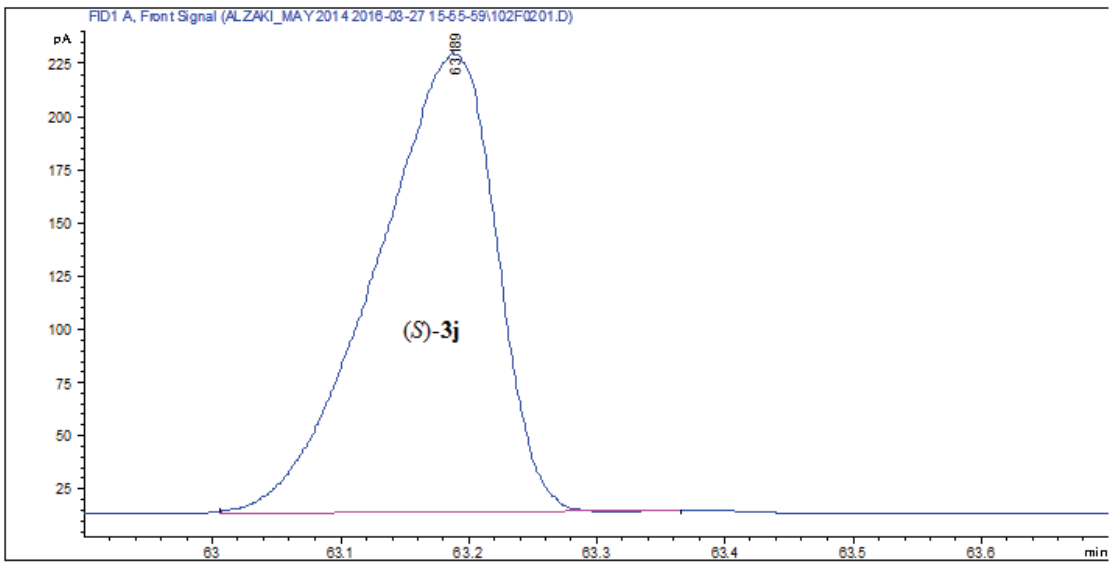
a)



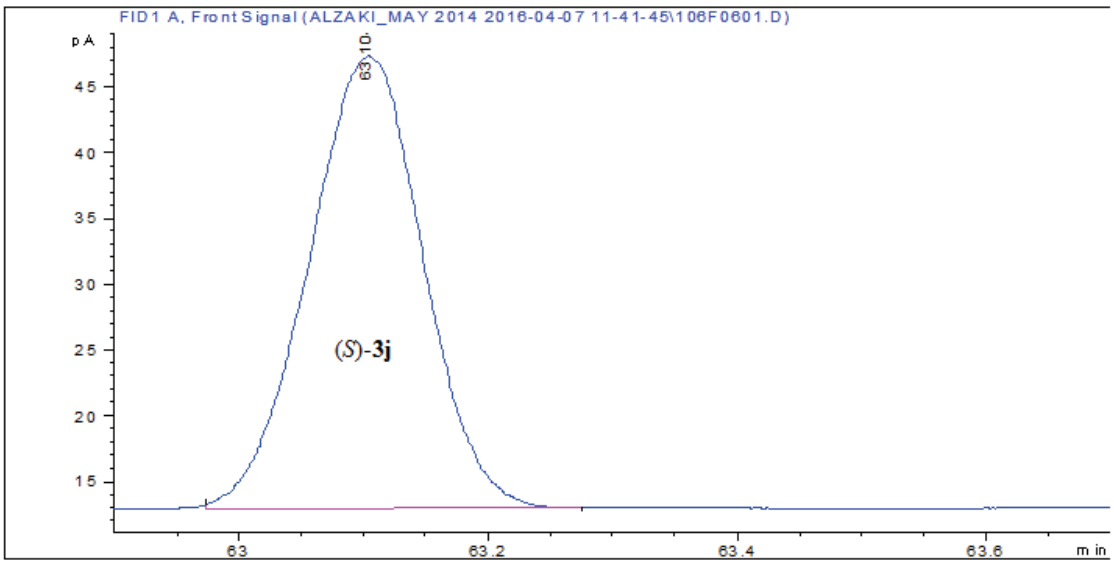
b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-27 15-55-59\101F0101.D)
pA
250
200
150
100
50
0
63.207
(S)-3j
63
63.1
63.2
63.3
63.4
63.5
63.6
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-27 15-55-59\102F0201.D)
pA
225
200
175
150
125
100
75
50
25
63.189
(S)-3j
63
63.1
63.2
63.3
63.4
63.5
63.6
min

d)



e)

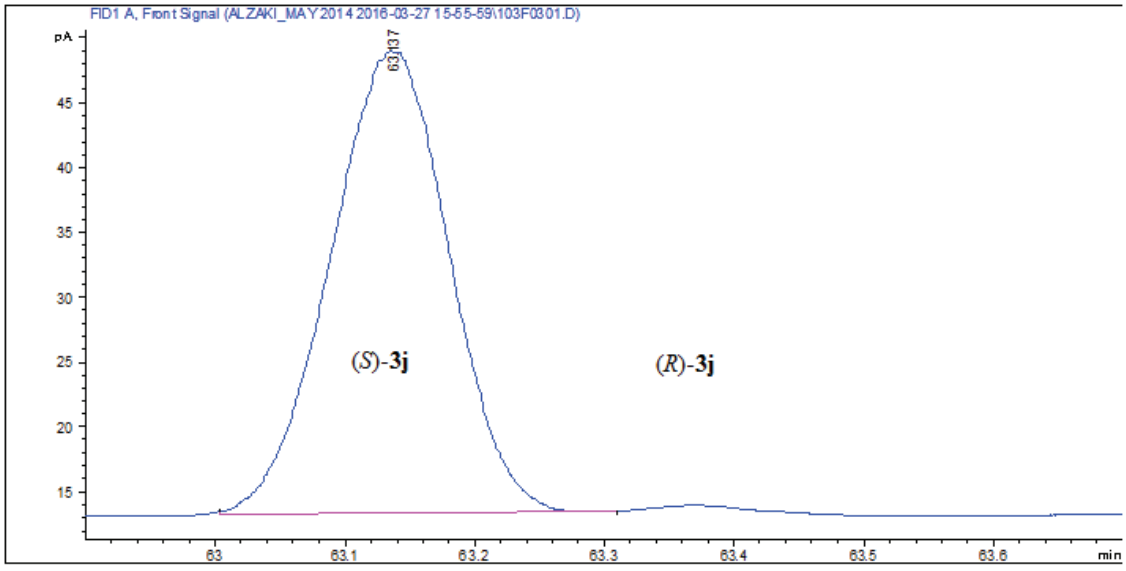
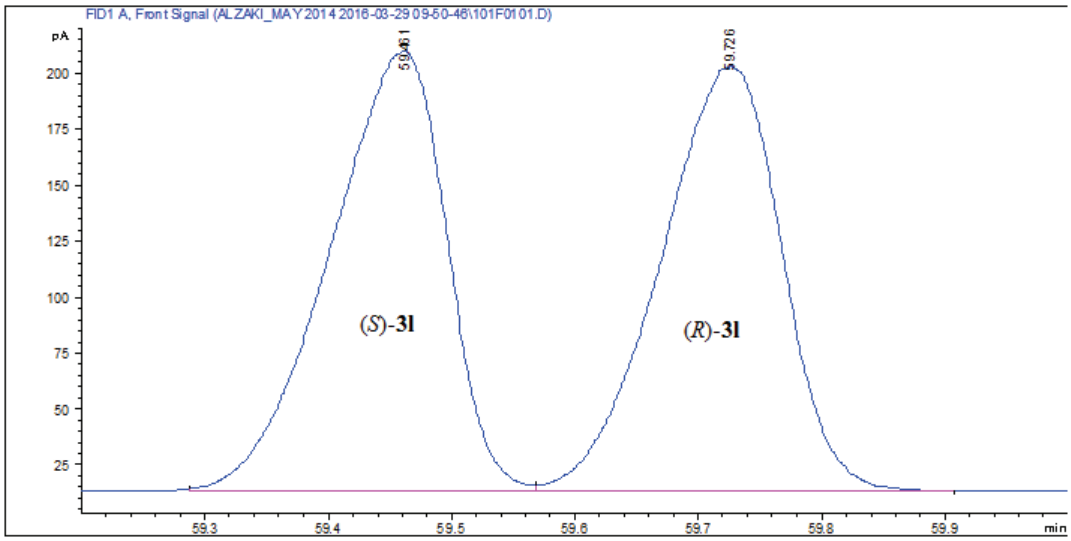
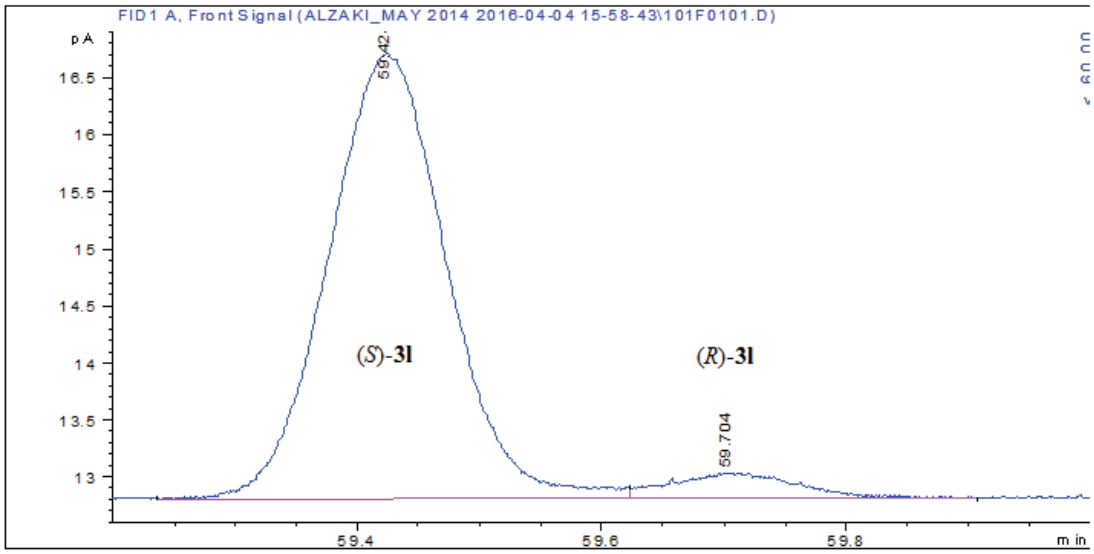


Figure A9. GC Chromatogram of: a) acetate derivative of *rac*-**2j** (made by reduction of **1j**with $NaBH_4$), b) acetate derivative of (*S*)-**2j** W110G TeSADH, c) acetate derivative of (*S*)-**2j** W110A TeSADH, d) acetate derivative of (*S*)-**2j** W110V TeSADH, d) acetate derivative of (*S*)-**2j** W110A/I86A TeSADH.

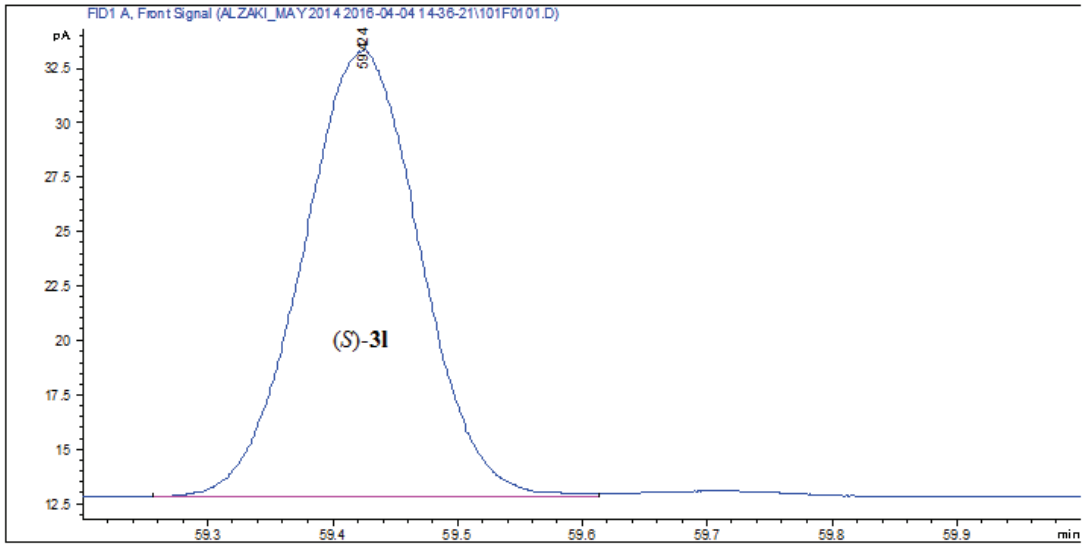
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-29 09-50-46\101F0101.D)
pA
200
175
150
125
100
75
50
25
59.461
59.726
(S)-3l
(R)-3l
59.3
59.4
59.5
59.6
59.7
59.8
59.9
min

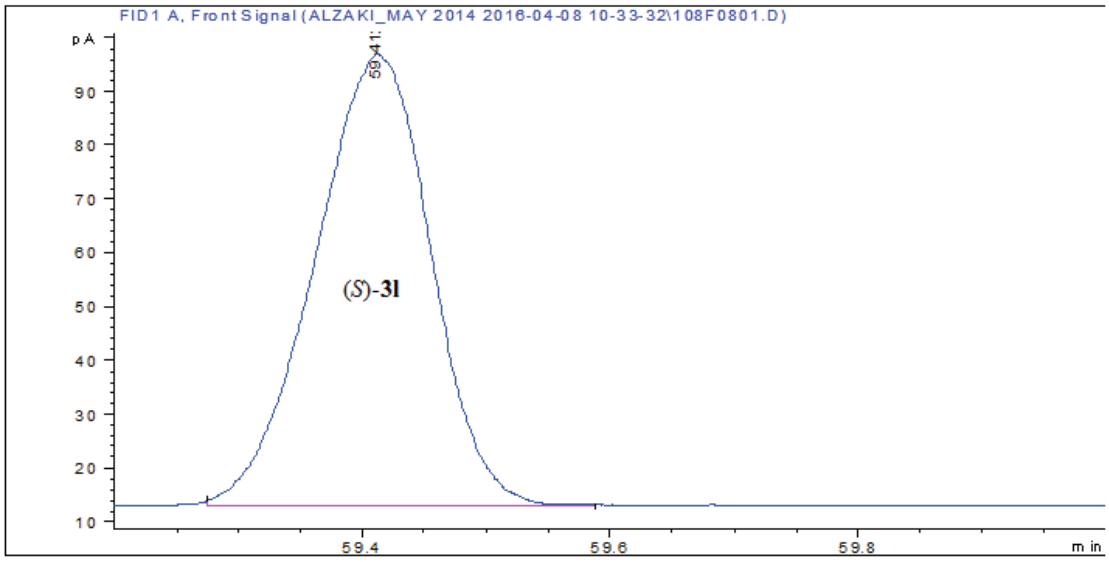
b)

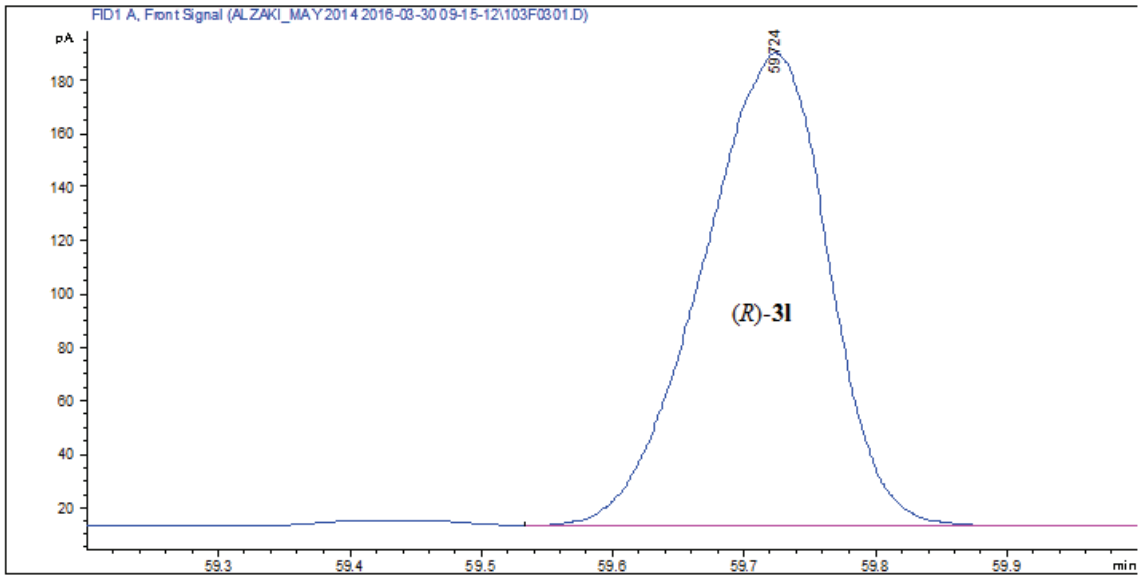

FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-04 15-58-43\101F0101.D)
pA
16.5
16
15.5
15
14.5
14
13.5
13
59.42
(S)-3l
(R)-3l
59.704
59.4
59.6
59.8
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-04 14-36-21\101F0101.D)
pA
59.24
(S)-3l
min

d)

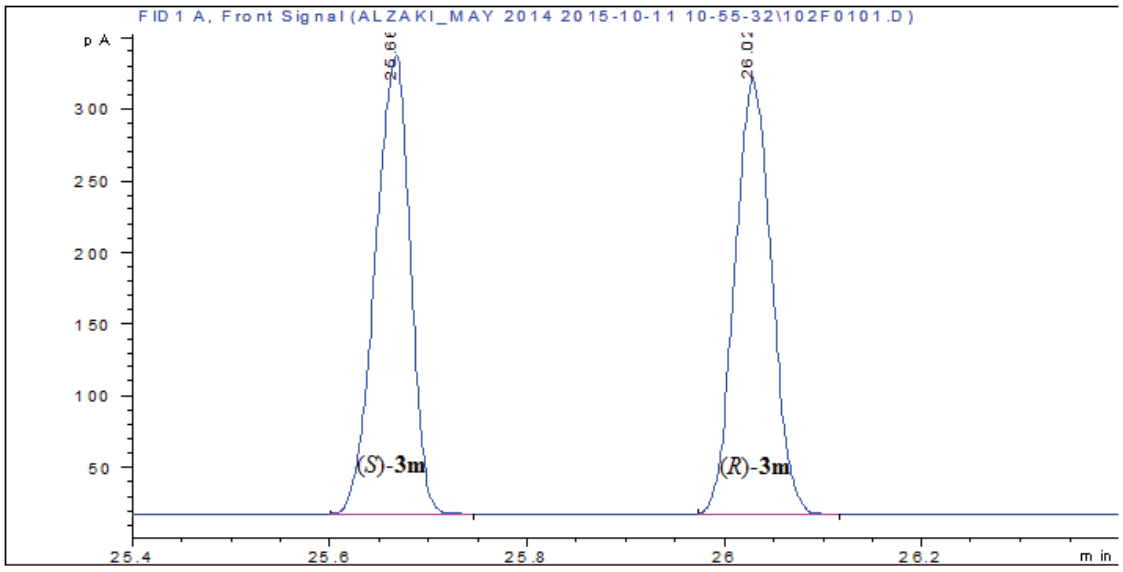

FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-08 10-33-32\108F0801.D)
pA
59.41
(S)-3l
min



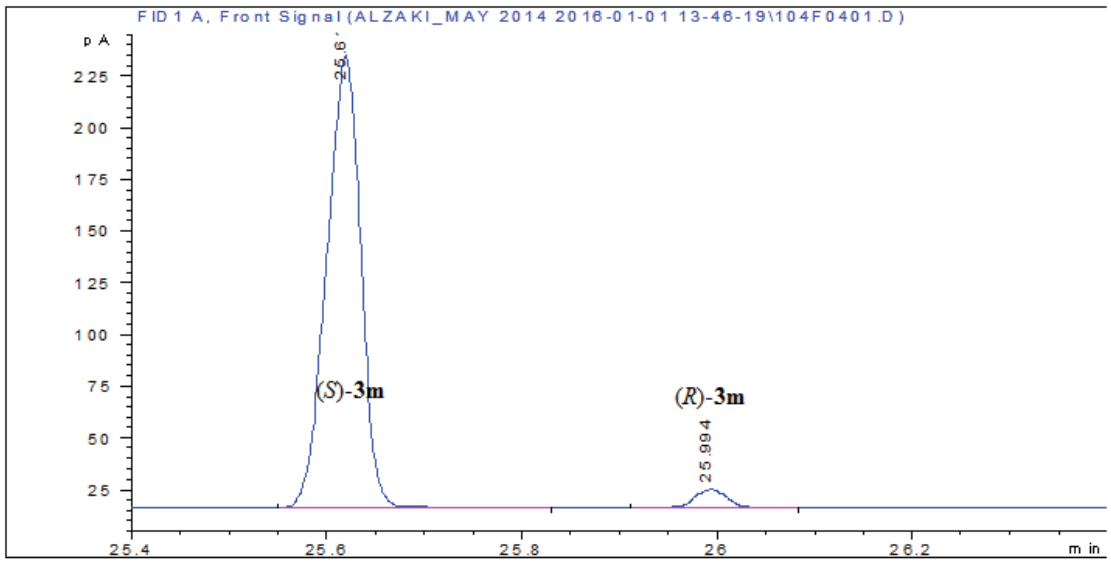
e)

Figure A10. GC Chromatogram of: a) acetate derivative of *rac*-**2l** (made by reduction of **1l** with $NaBH_4$), b) acetate derivative of (*S*)-**2l** W110G TeSADH, c) acetate derivative of (*S*)-**2l** W110A *TeSADH*, d) acetate derivative of (*S*)-**2l** W110VTeSADH, e) acetate derivative of (*R*)-**2l** W110A/I86A TeSADH.

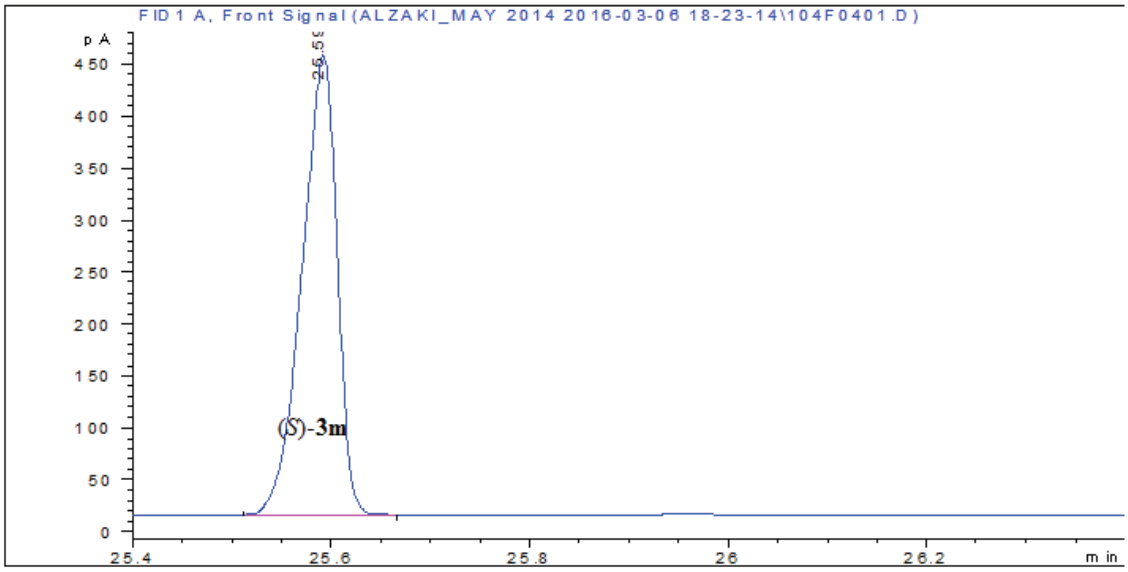
a)



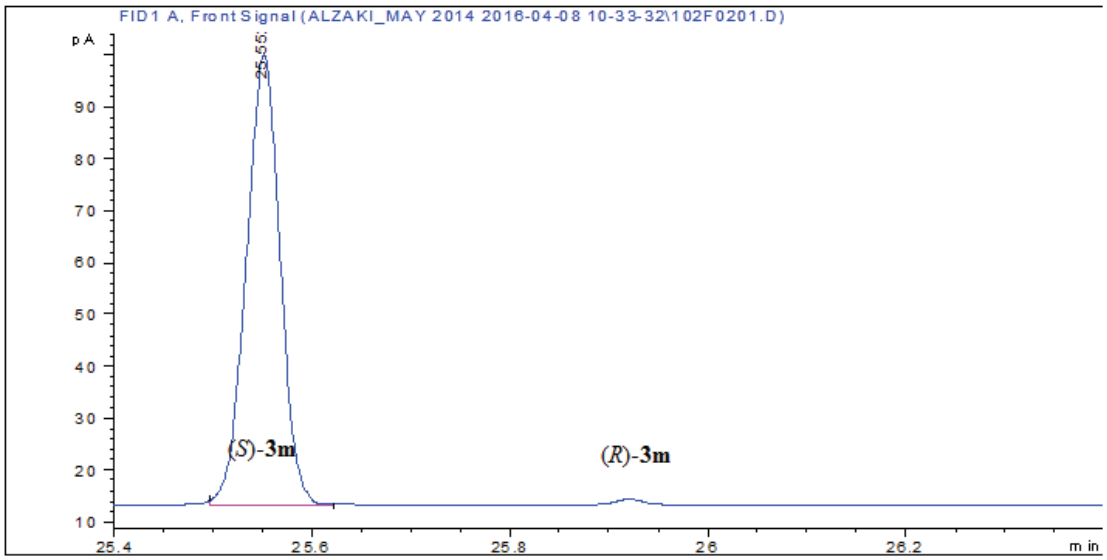
b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-01-01 13-46-19\104F0401.D)
pA
225
200
175
150
125
100
75
50
25
25.6
(S)-3m
(R)-3m
25.994
25.4
25.6
25.8
26
26.2
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-06 18-23-14\104F0401.D)
pA
450
400
350
300
250
200
150
100
50
0
25.5
(S)-3m
25.4
25.6
25.8
26
26.2
min

d)



e)

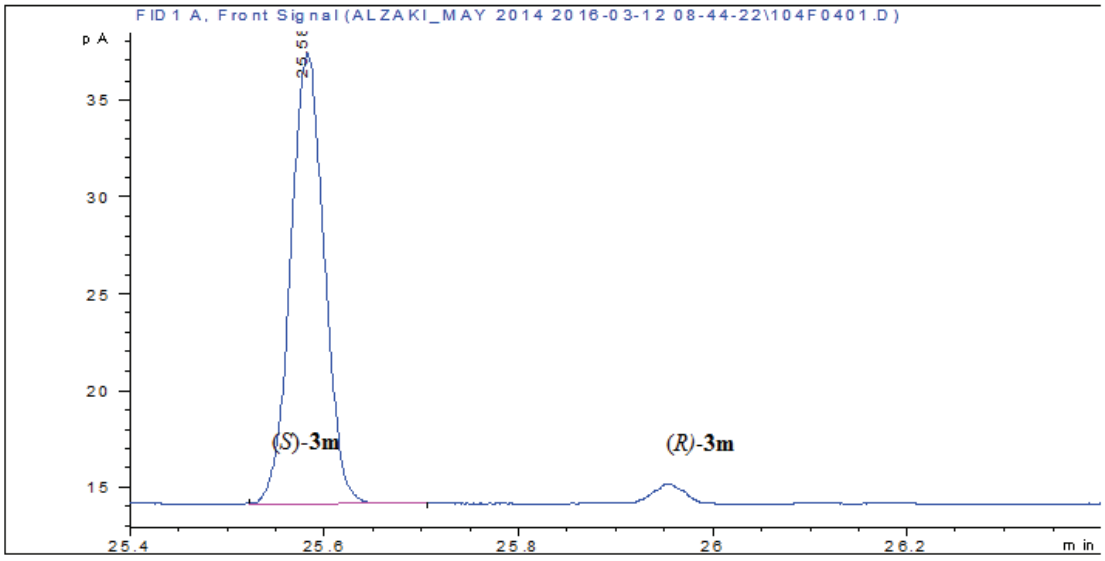
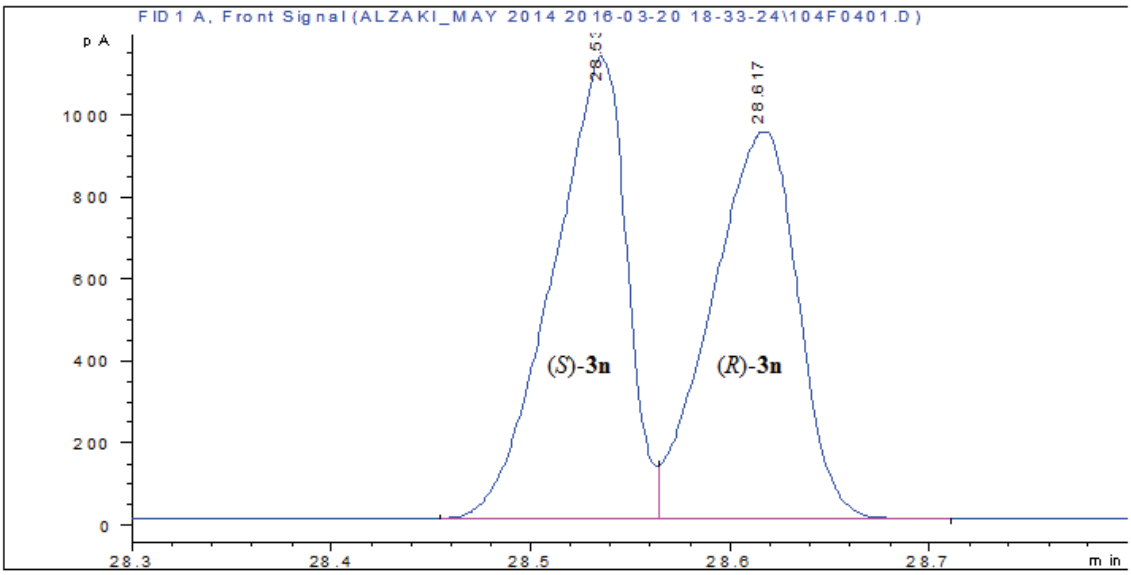
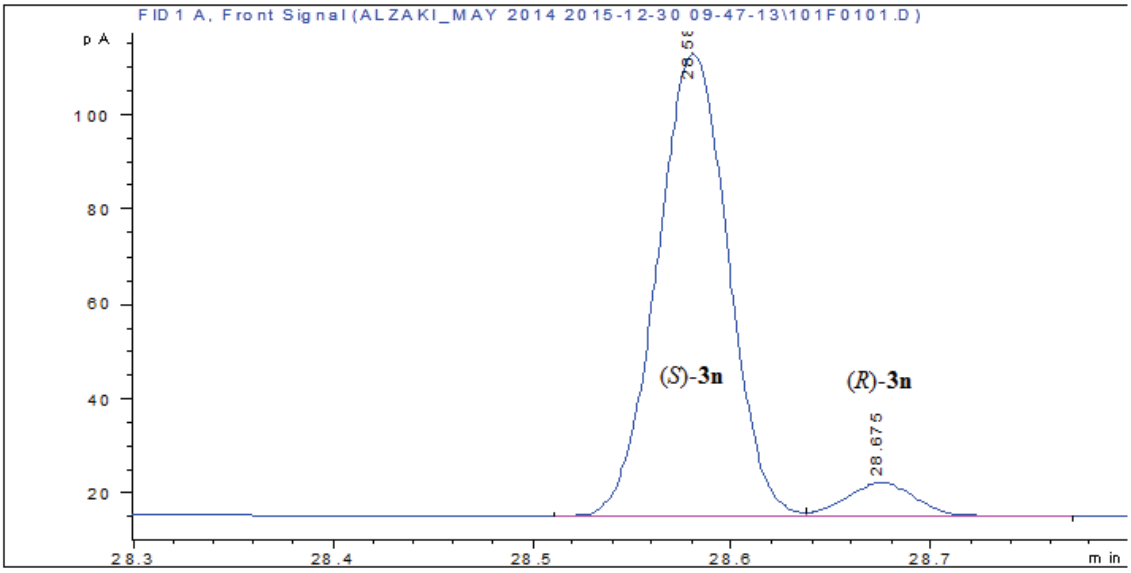


Figure A11. GC Chromatogram of: a) acetate derivative of *rac*-**2m** (made by reduction of **1m** with $NaBH_4$), b) acetate derivative of (*S*)-**2m** W110G TeSADH, c) acetate derivative of (*S*)-**2m** W110A TeSADH, d) acetate derivative of (*S*)-**2m** W110V TeSADH, e) acetate derivative of (*R*)-**2m** W110A/I86A TeSADH.

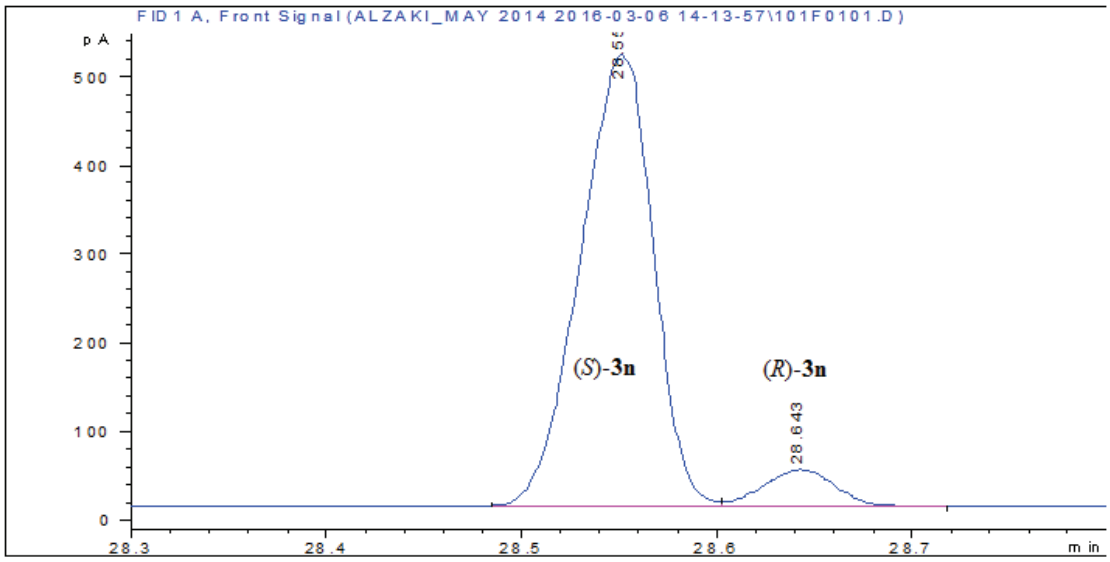
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-20 18-33-24\104F0401.D)
pA
1000
800
600
400
200
0
28.617
(S)-3n
(R)-3n
28.3
28.4
28.5
28.6
28.7
min

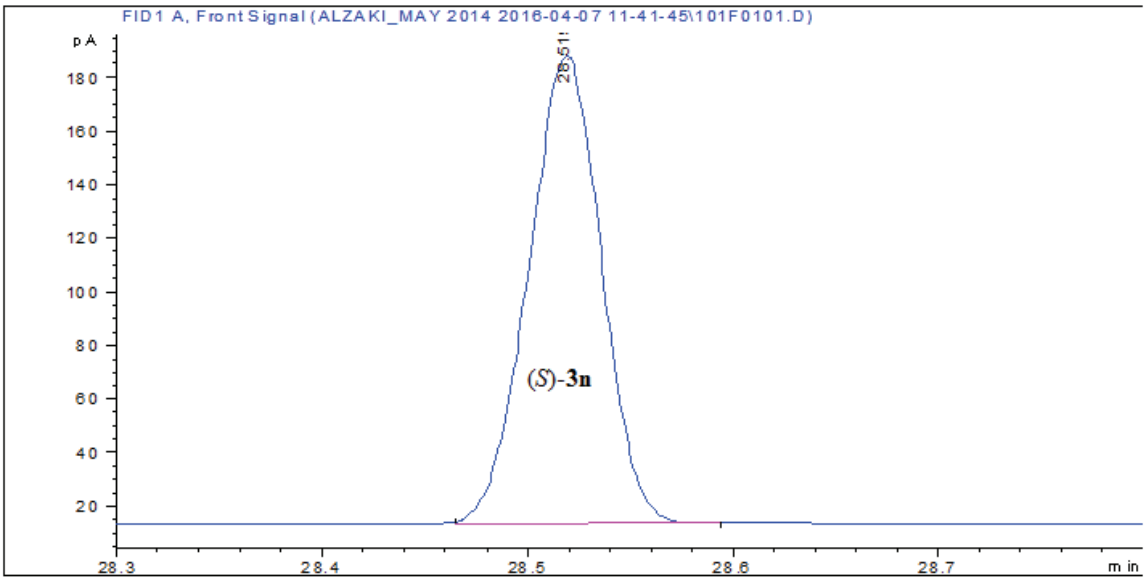
b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2015-12-30 09-47-13\101F0101.D)
pA
100
80
60
40
20
(S)-3n
(R)-3n
28.675
28.3
28.4
28.5
28.6
28.7
min

c)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-03-06 14-13-57\101F0101.D)
pA
500
400
300
200
100
0
28.643
(S)-3n
(R)-3n
28.3
28.4
28.5
28.6
28.7
min

d)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-07 11-41-45\101F0101.D)
pA
180
160
140
120
100
80
60
40
20
(S)-3n
28.3
28.4
28.5
28.6
28.7
min

e)

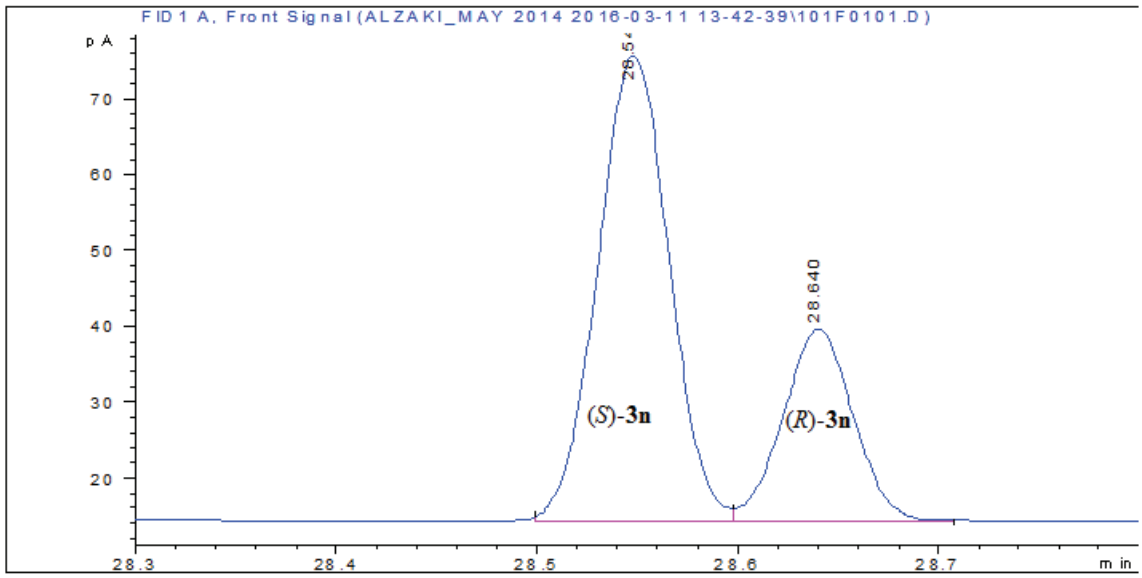
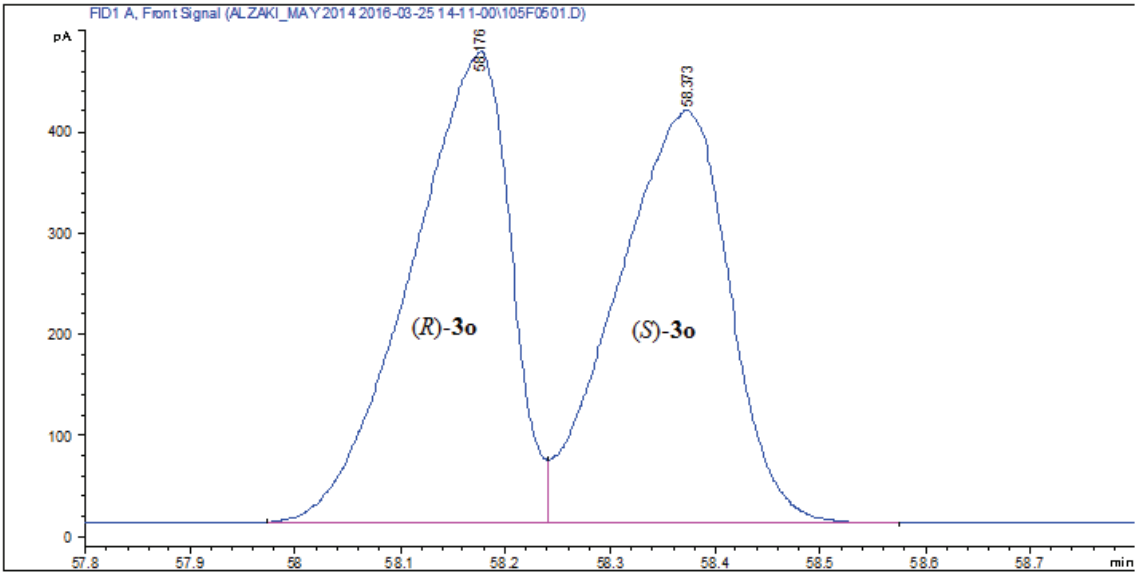
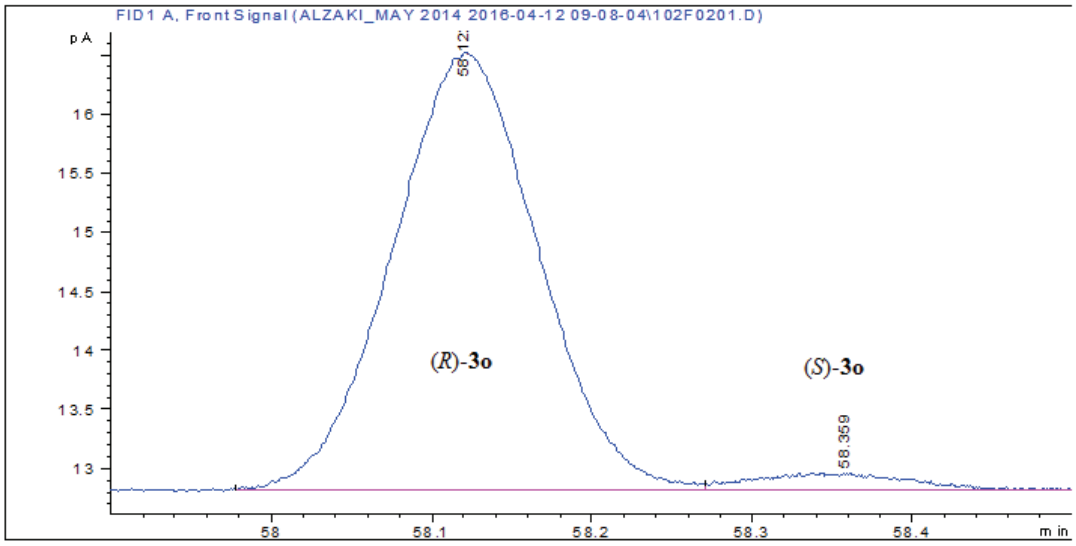


Figure A12. GC Chromatogram of: a) acetate derivative of *rac*-**2n** (made by reduction of **1n** with NaBH4), b) acetate derivative of (*S*)-**2n** W110G TeSADH, c) acetate derivative of (*S*)-**2n** W110A TeSADH, d) acetate derivative of (*S*)-**2n**with W110V TeSADH, e) acetate derivative of (*S*)-**2n** W110A/I86A TeSADH.

a)



b)



c)

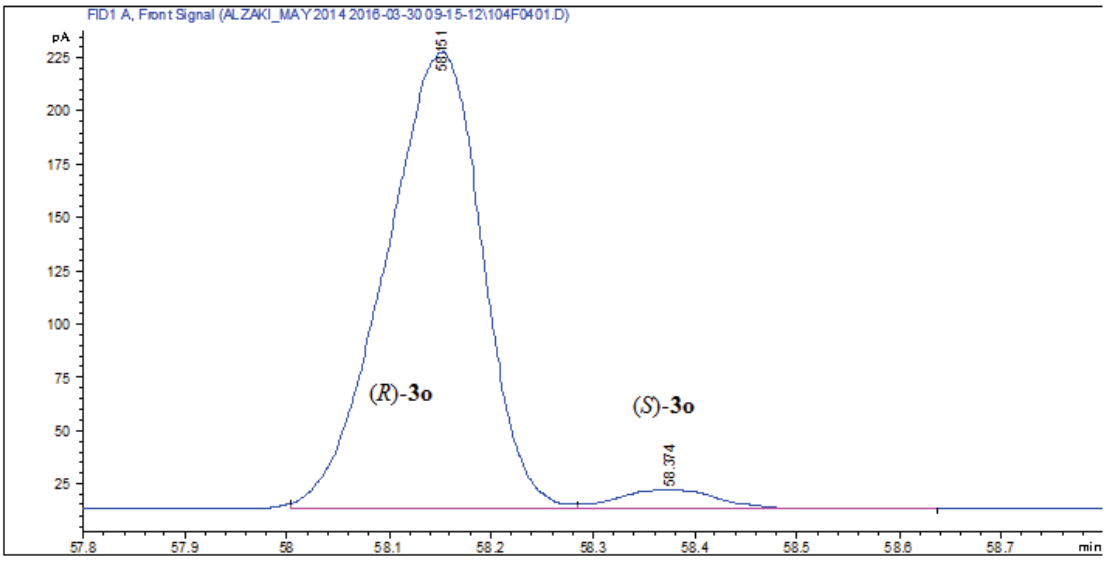
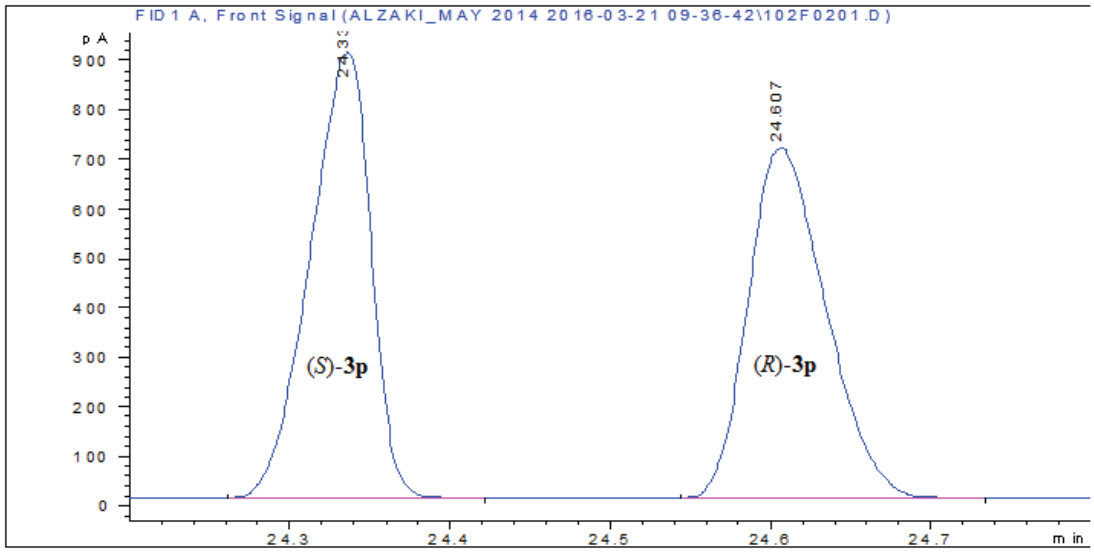


Figure A13. GC Chromatogram of: a) acetate derivative of *rac*-**2o** (made by reduction of **1o** with $NaBH_4$), b) acetate derivative of (*R*)-**2o** with W110V TeSADH, c) acetate derivative of (*R*)-**2o** with W110A/I86A TeSADH.

a)



b)

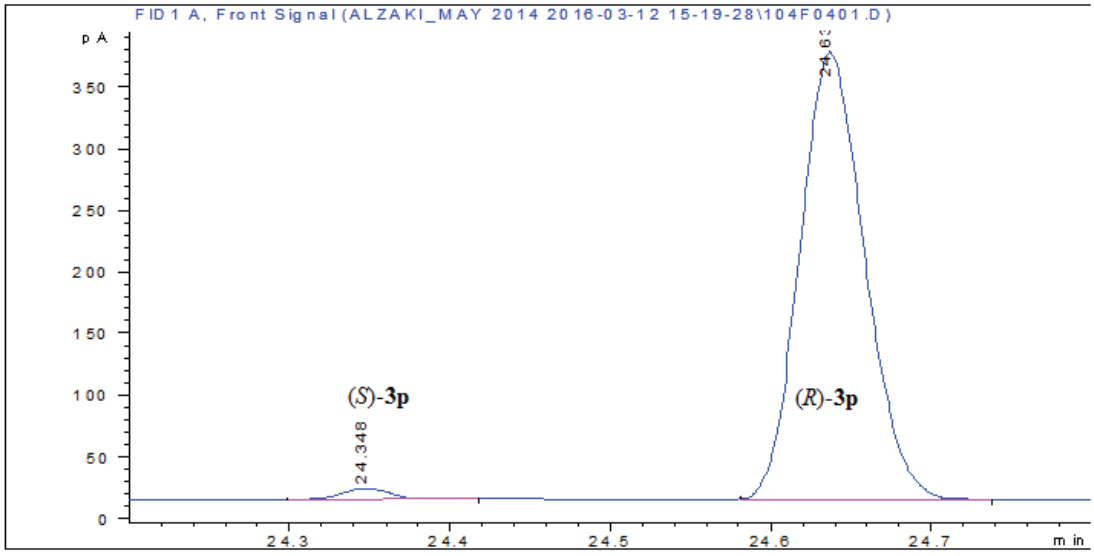
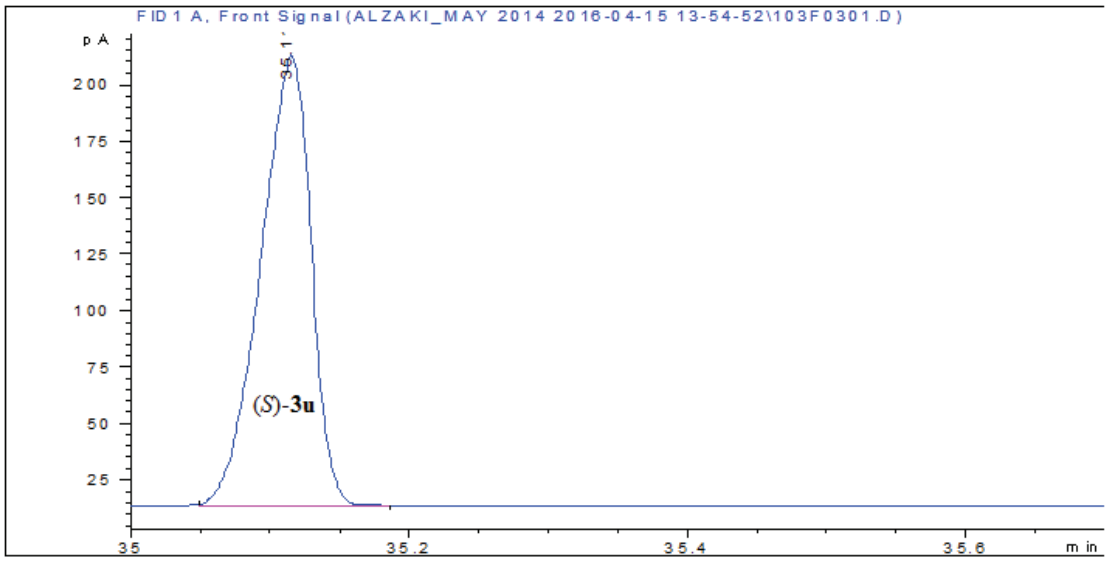
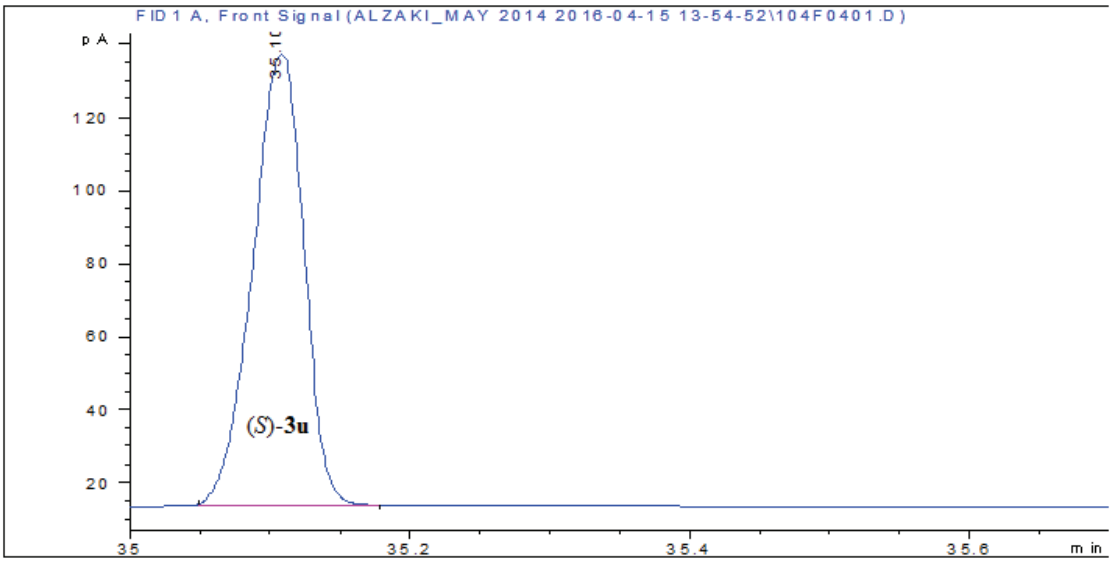


Figure A14. GC Chromatogram of: a) acetate derivative of *rac*-**2k** (made by reduction of **1p** with NaBH4), b) acetate derivative of (*R*)-**2p** with W110A/I86A TeSADH.

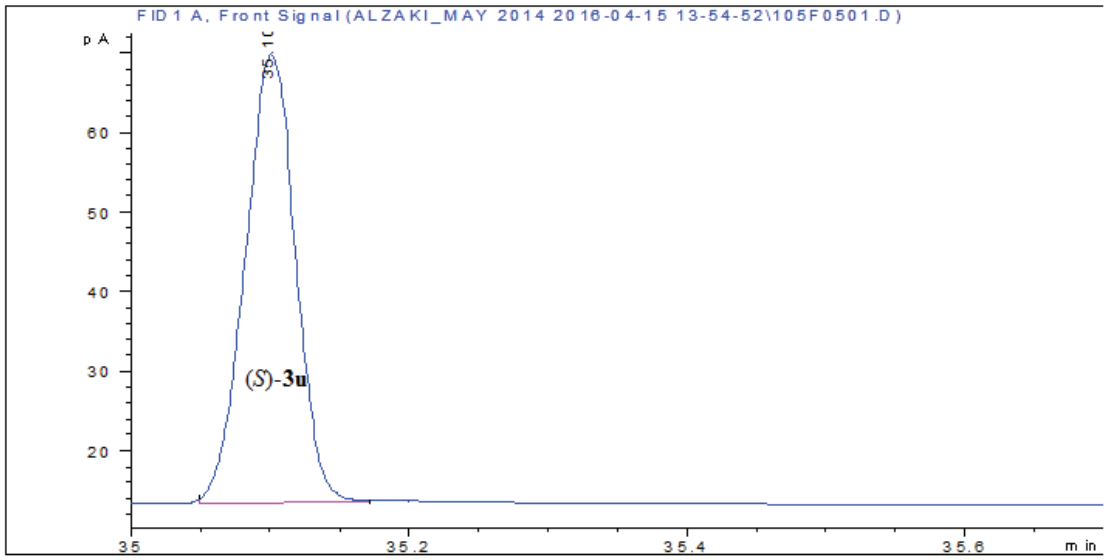
a)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-15 13-54-52\103F0301.D)
pA
200
175
150
125
100
75
50
25
35.1
(S)-3u
35
35.2
35.4
35.6
min

b)


FID1 A, Front Signal (ALZAKI_MAY 2014 2016-04-15 13-54-52\104F0401.D)
pA
120
100
80
60
40
20
35.1
(S)-3u
35
35.2
35.4
35.6
min

c)



d)

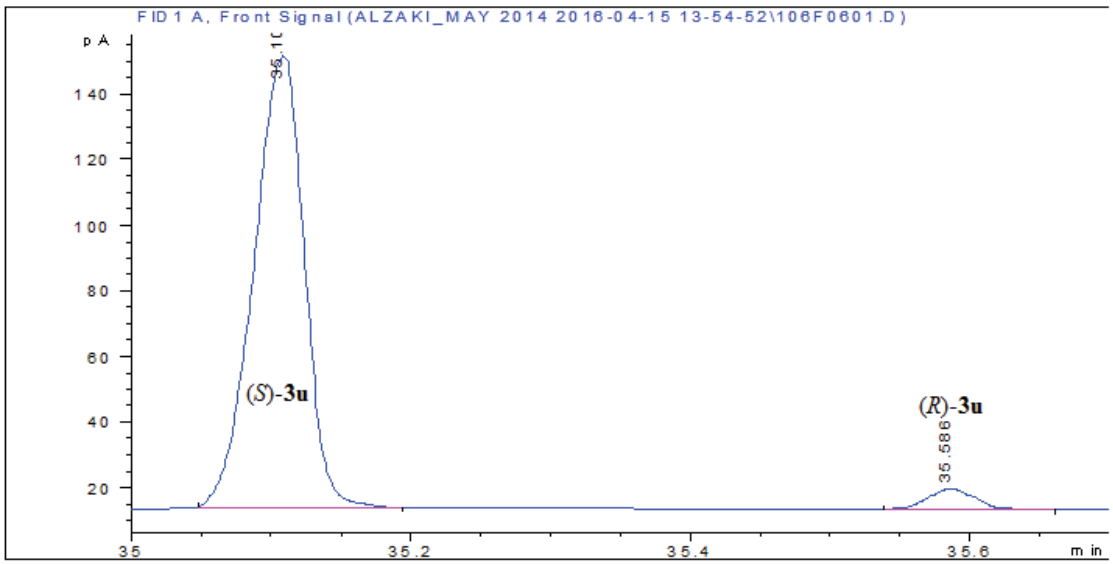
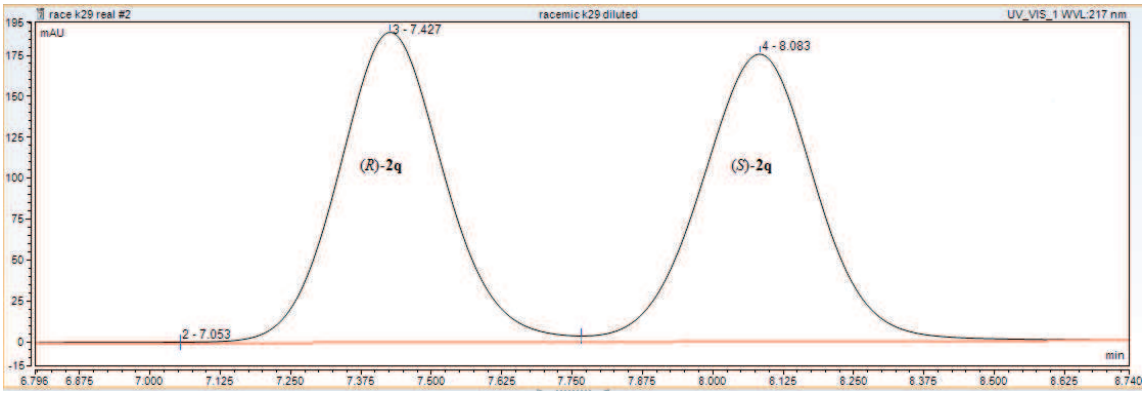


Figure A15. GC Chromatogram of: a) acetate derivative of (*S*)-**2u** W110G TeSADH, b) acetate derivative of (*S*)-**2u** W110A TeSADH, c) acetate derivative of (*S*)-**2u** with W110V TeSADH, d) acetate derivative of (*S*)-**2u** W110A/I86A TeSADH.

a)



b)

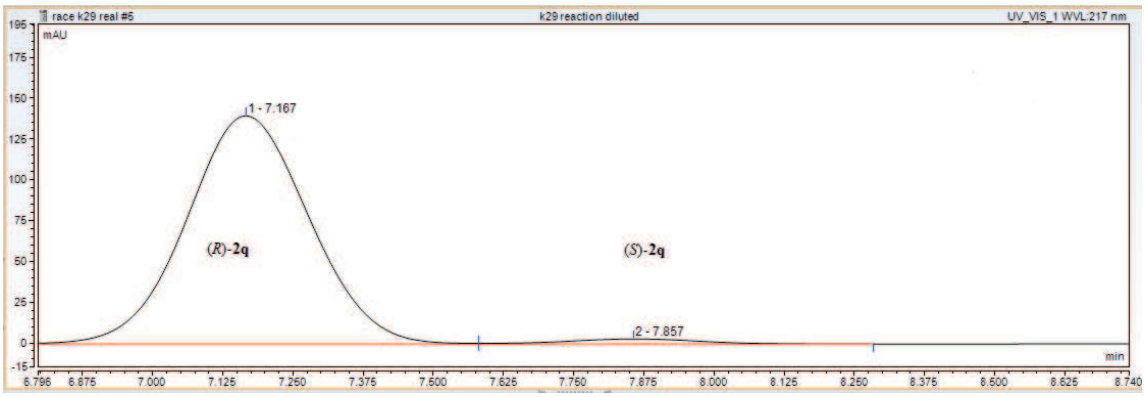
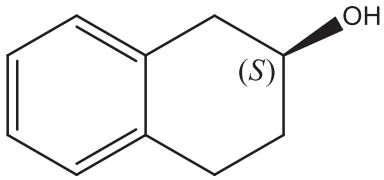


Figure A16. HPLC Chromatogram of: a) *rac*-**2q** (made by reduction of **1q**with $NaBH_4$), b) (*R*)-**2q** with W110A/I86A TeSADH.

2. Mass spectra



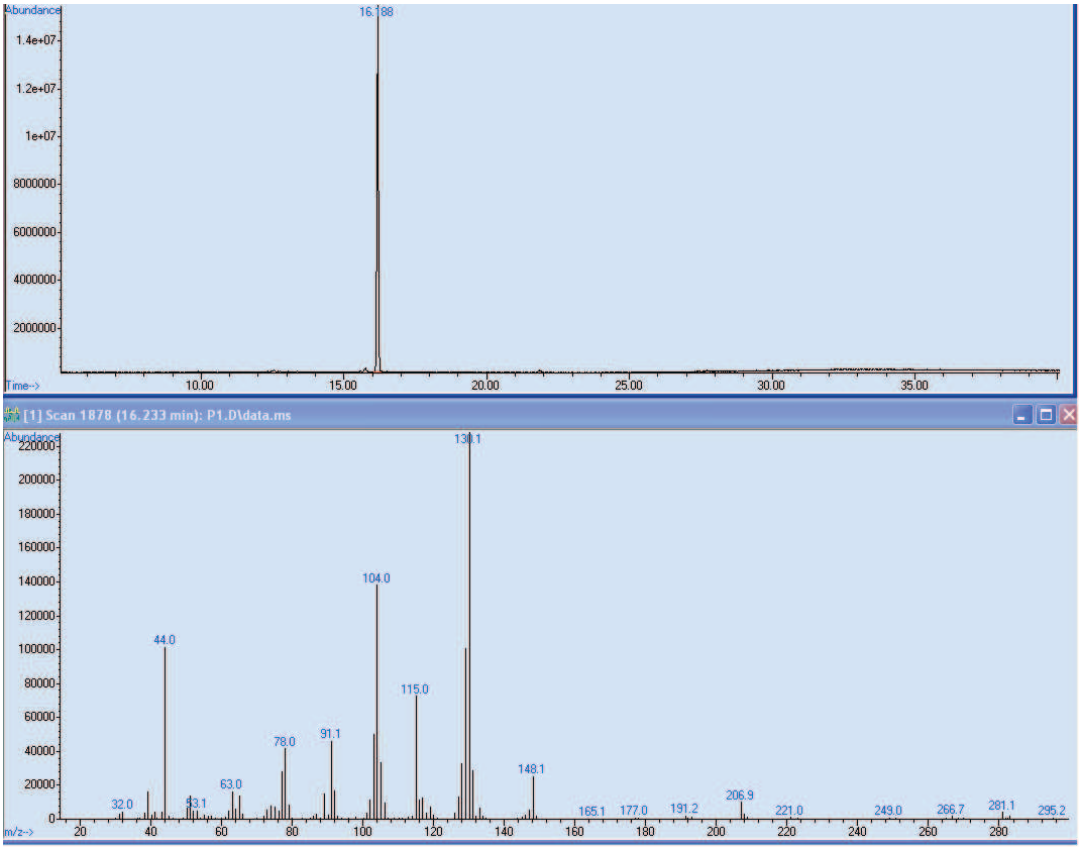
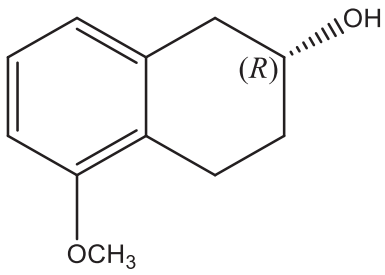


Figure A17. GC chromatogram and MS spectrum of the (*S*)-**2a** prepared by W110G TeSADH.



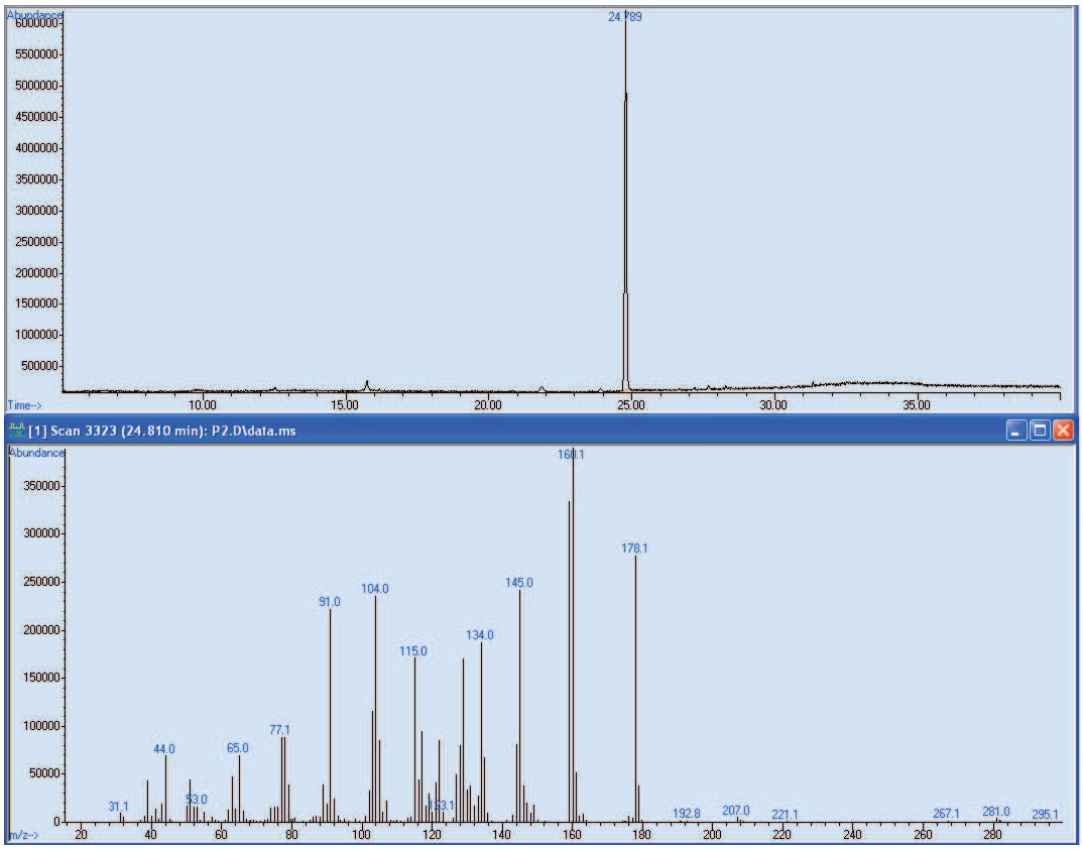
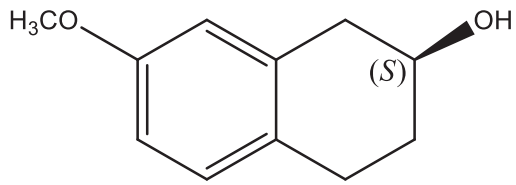


Figure A18. GC chromatogram and MS spectrum of the (*R*)-**2b** prepared by W110G TeSADH.



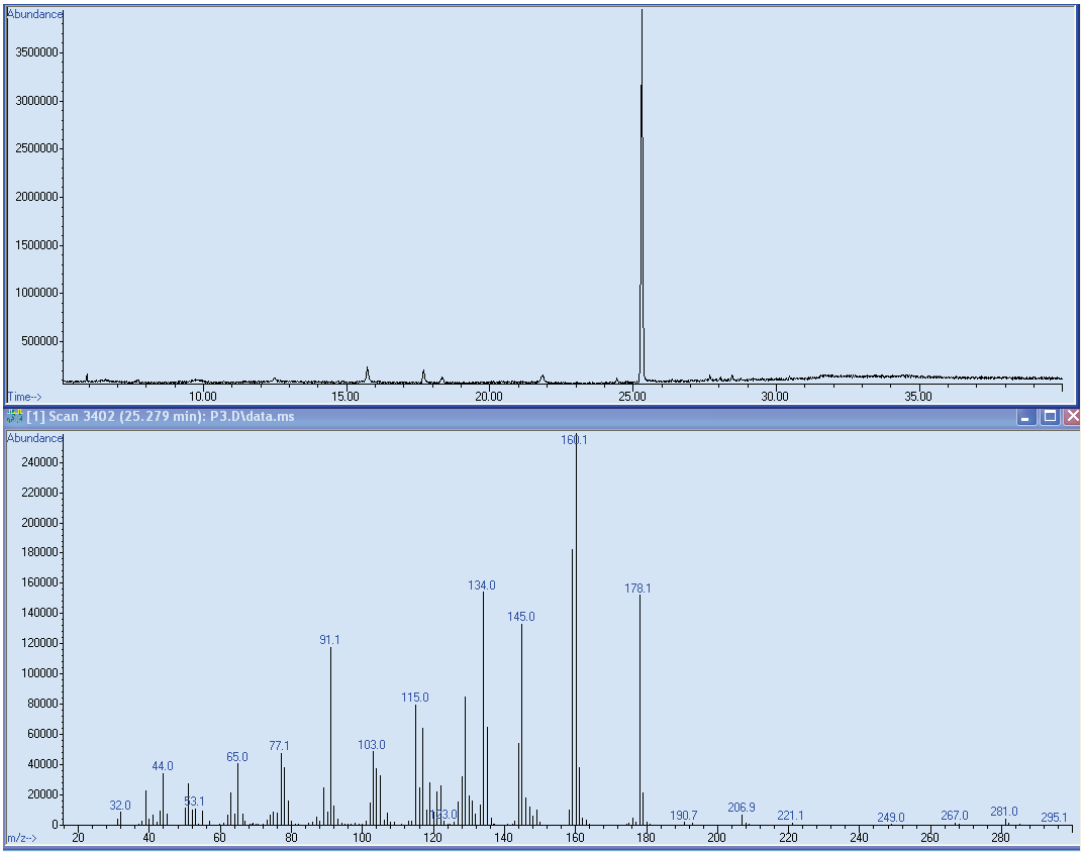
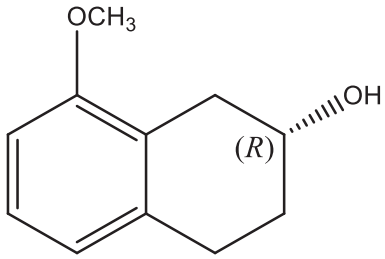


Figure A19. GC chromatogram and MS spectrum of the (*S*)-**2c** prepared by W110G TeSADH.



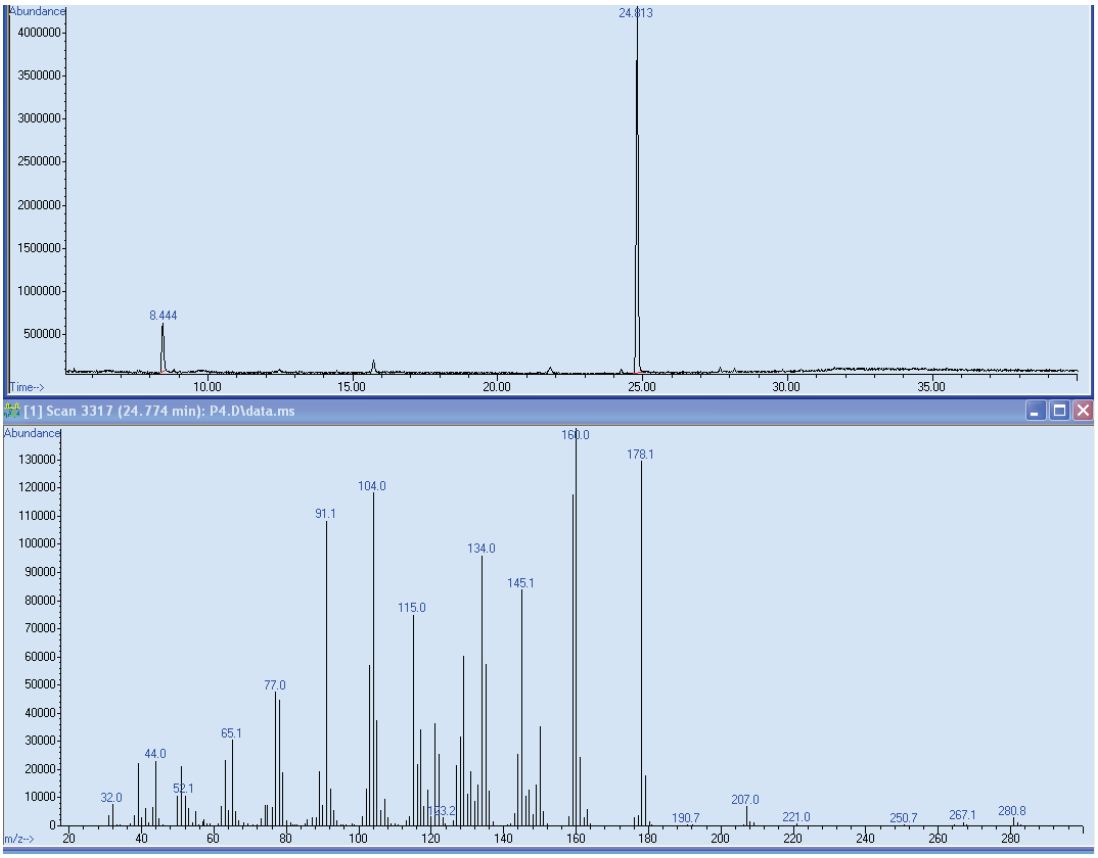
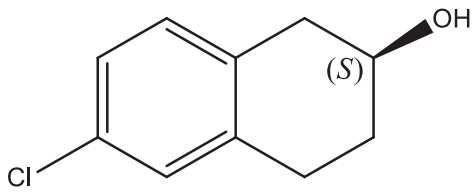


Figure A20. GC chromatogram and MS spectrum of the (*R*)-**2d** prepared by W110G TeSADH.



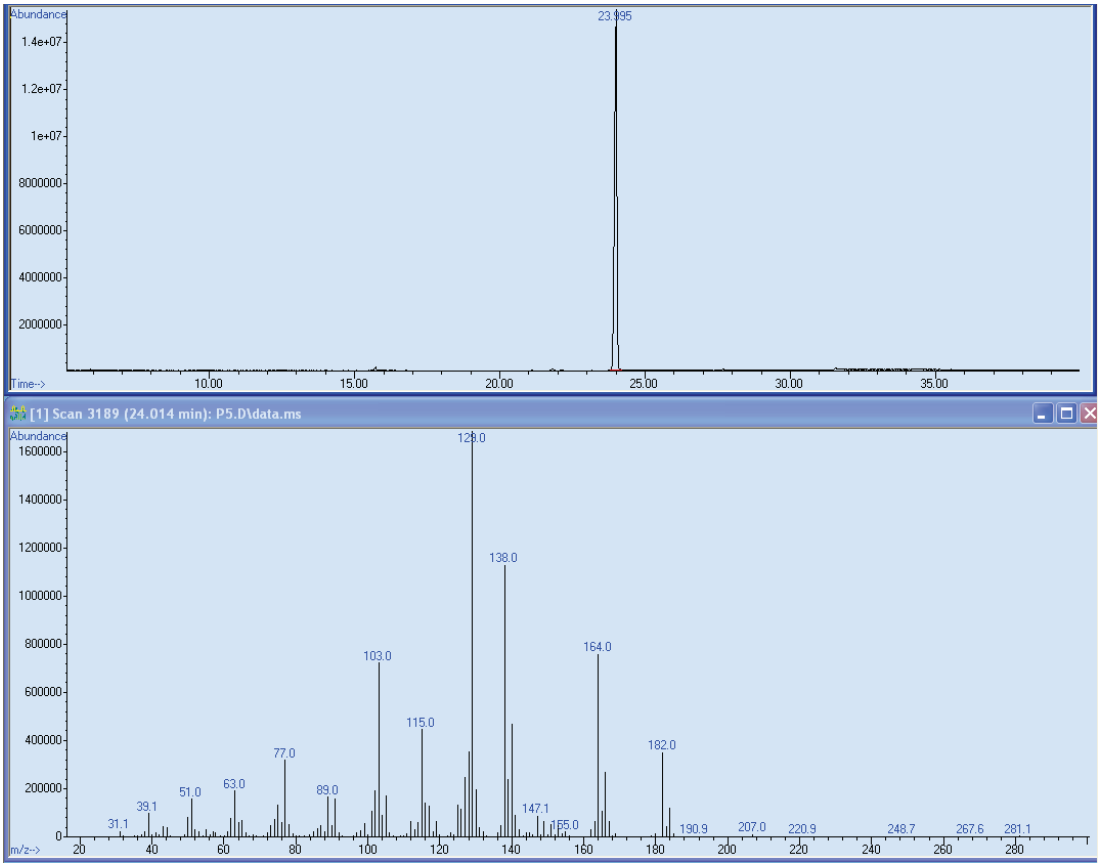
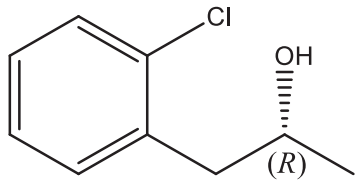


Figure A21. GC chromatogram and MS spectrum of the (*S*)-**2e** prepared by W110G TeSADH.



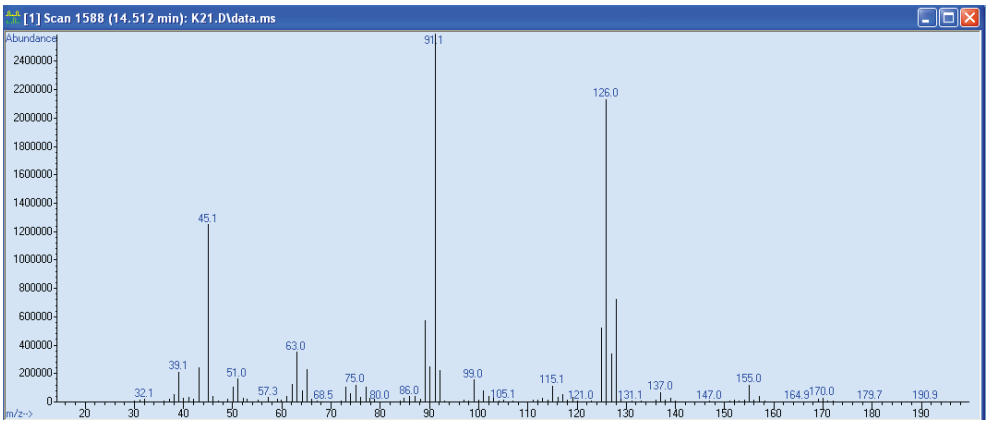
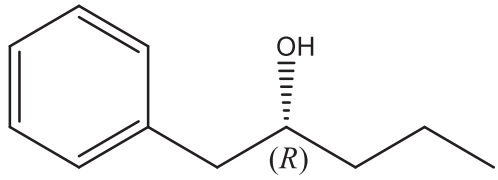


Figure A22. MS spectrum of the (*R*)-**2l** prepared by W110A/I86A TeSADH .



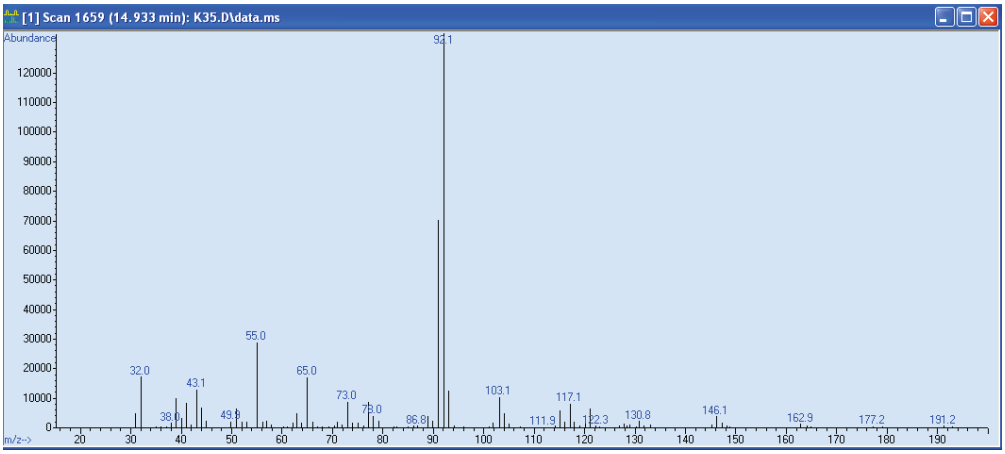


Figure A23. GC spectrum of the (*R*) -**2o** prepared by W110A/I86A TeSADH.

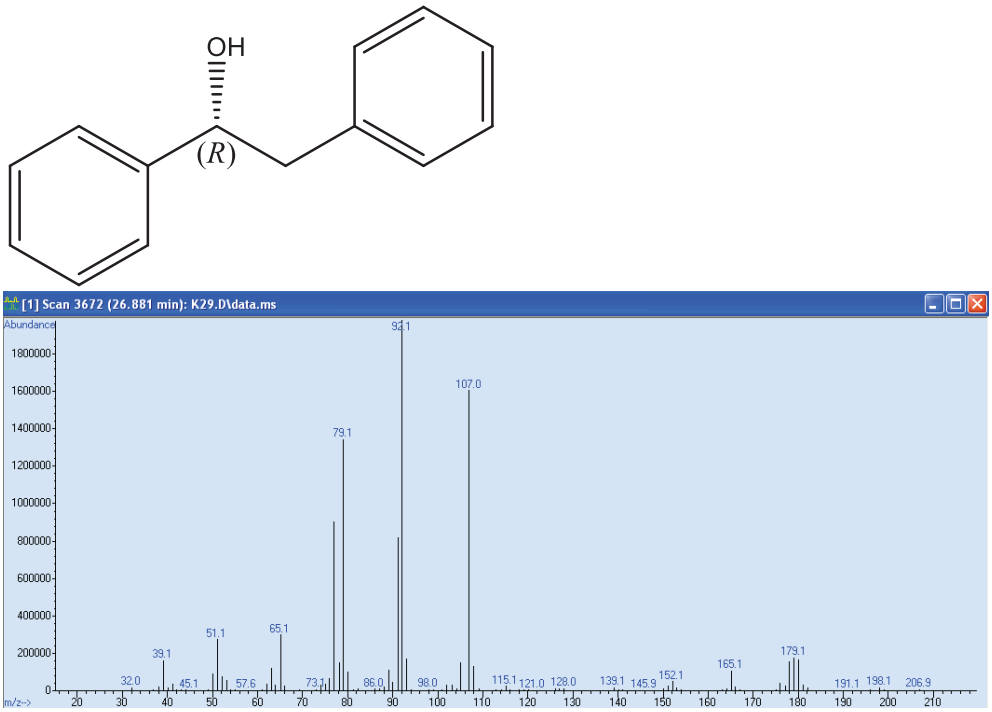


Figure A24. MS spectrum of the (*R*)-**2q** prepared by W110A/I86A TeSADH .

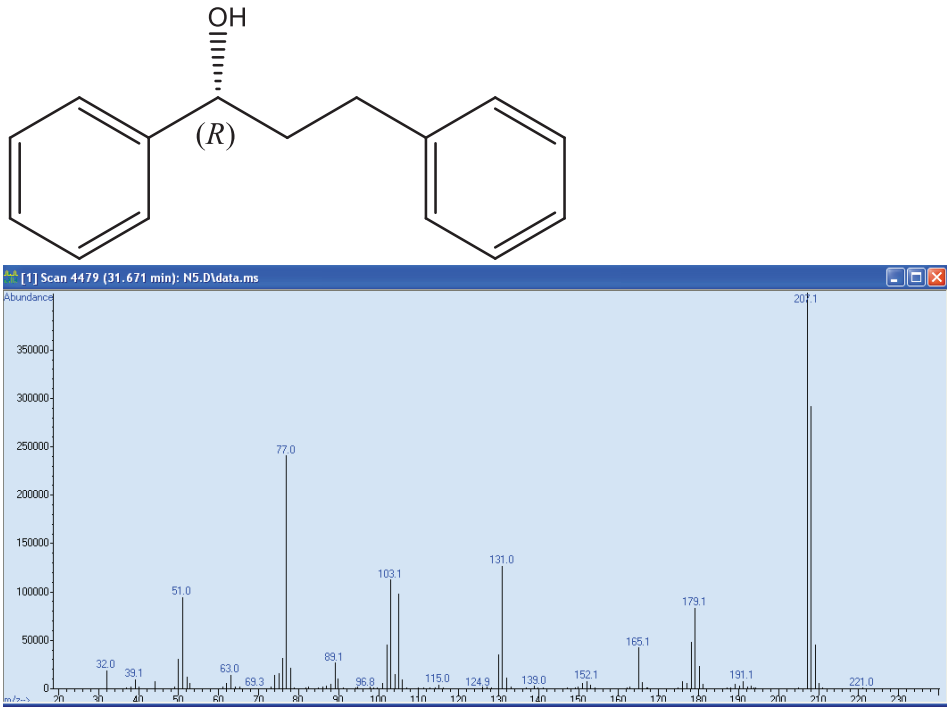
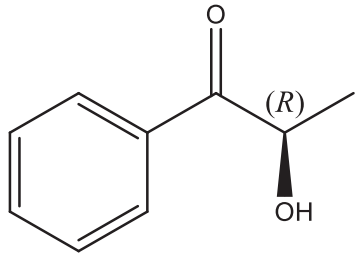
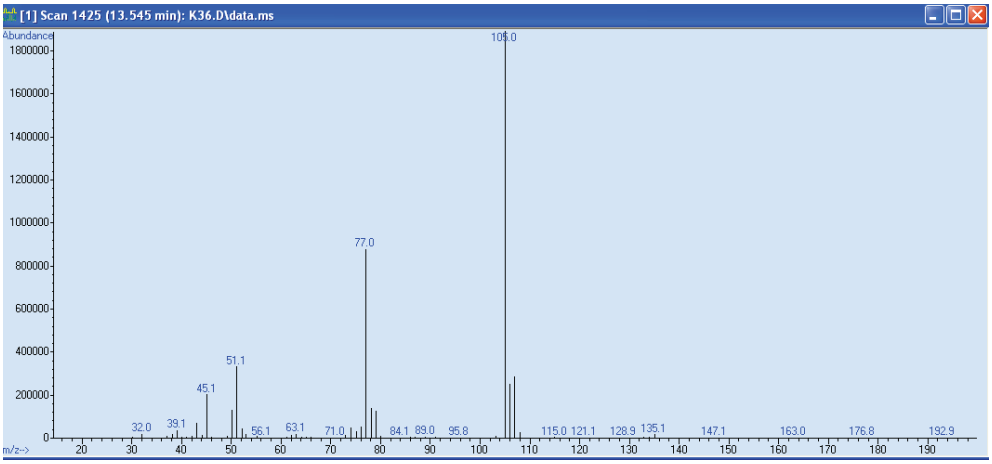
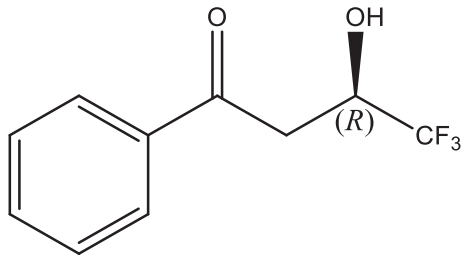


Figure A25. MS spectrum of the **2r** prepared by W110A/I86A TeSADH .





. **Figure A26**. MS spectrum of the (*R*)-**2s** prepared by W110A/I86A TeSADH.



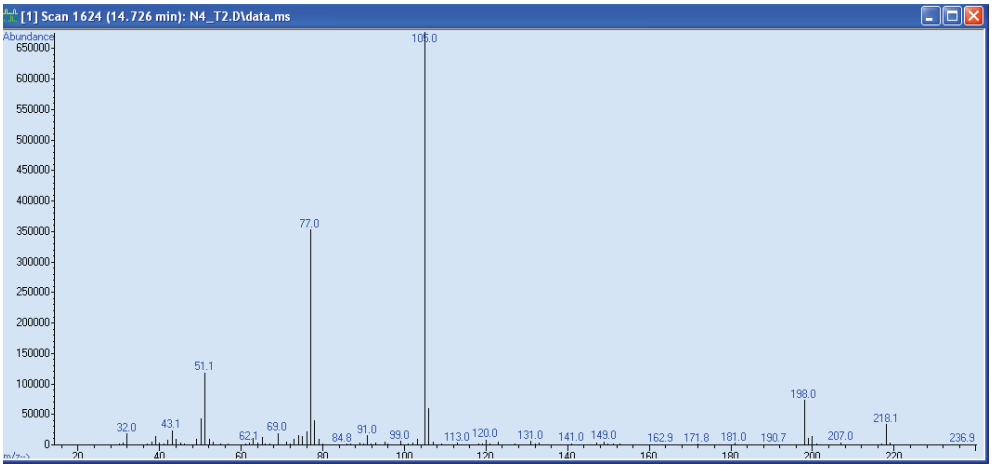
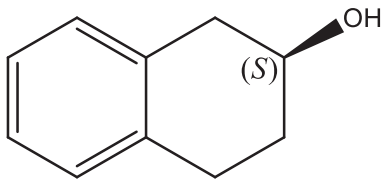


Figure A27. MS spectrum of the (*R*)-**2v** prepared by W110A/I86A TeSADH.

3. NMR spectra



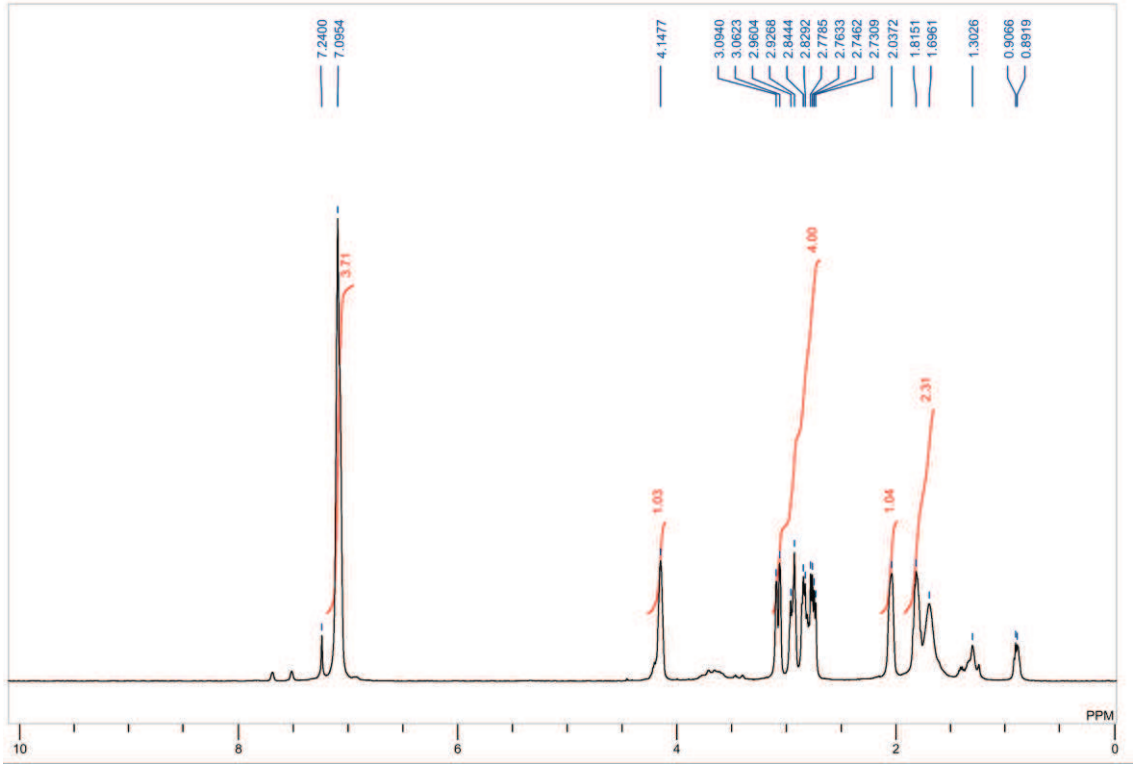


Figure A28. ^{1}H NMR spectrum of the (*S*)-**2a** prepared by W110G TeSADH.

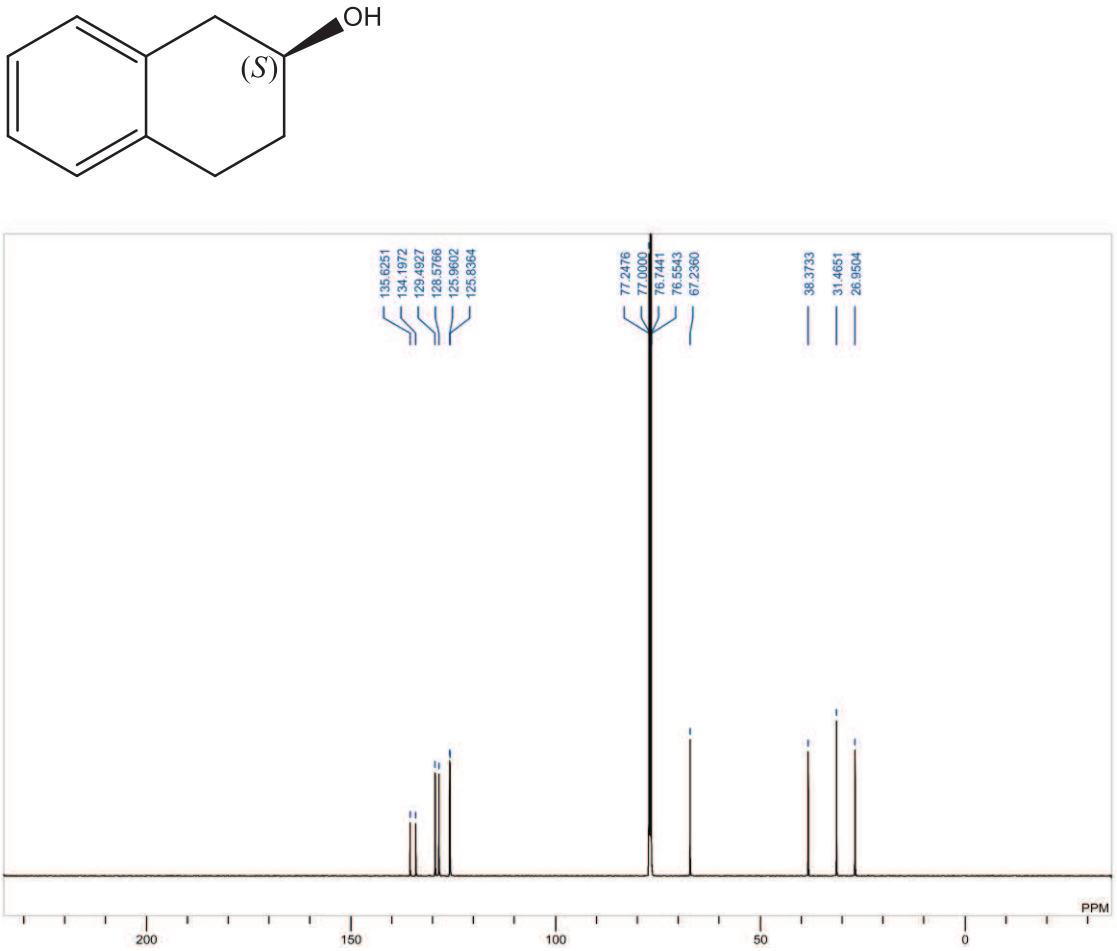


Figure A29. ^{13}C NMR spectrum of the (*S*)-**2a** prepared by W110G TeSADH.

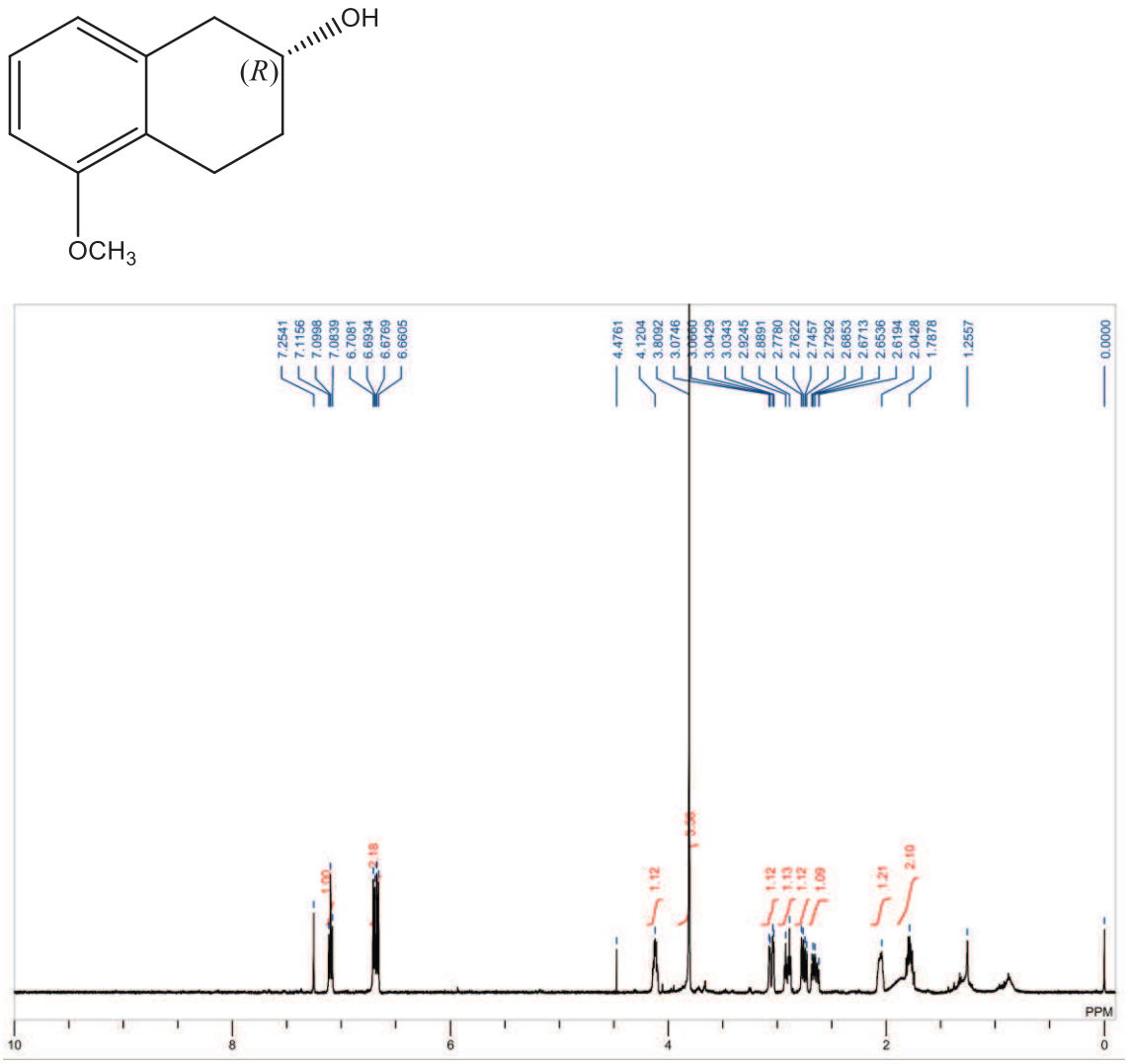


Figure A30. ^{1}H NMR spectrum of the **(*R*)-2b** prepared by W110G TeSADH.

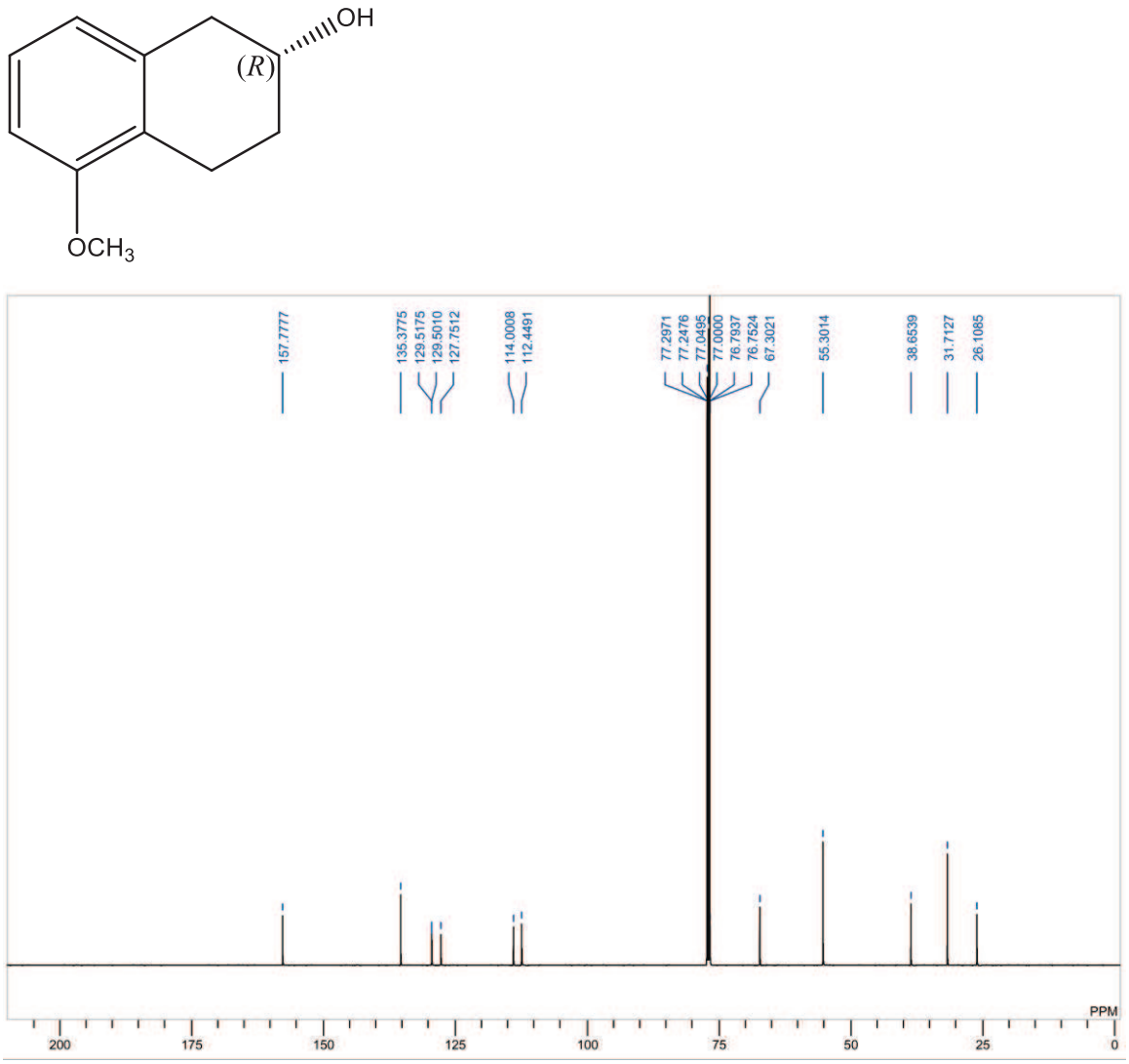
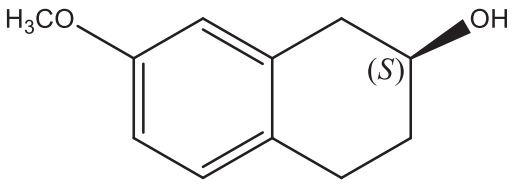


Figure A31. ^{13}C NMR spectrum of the (*R*)-**2b** prepared by W110G TeSADH.



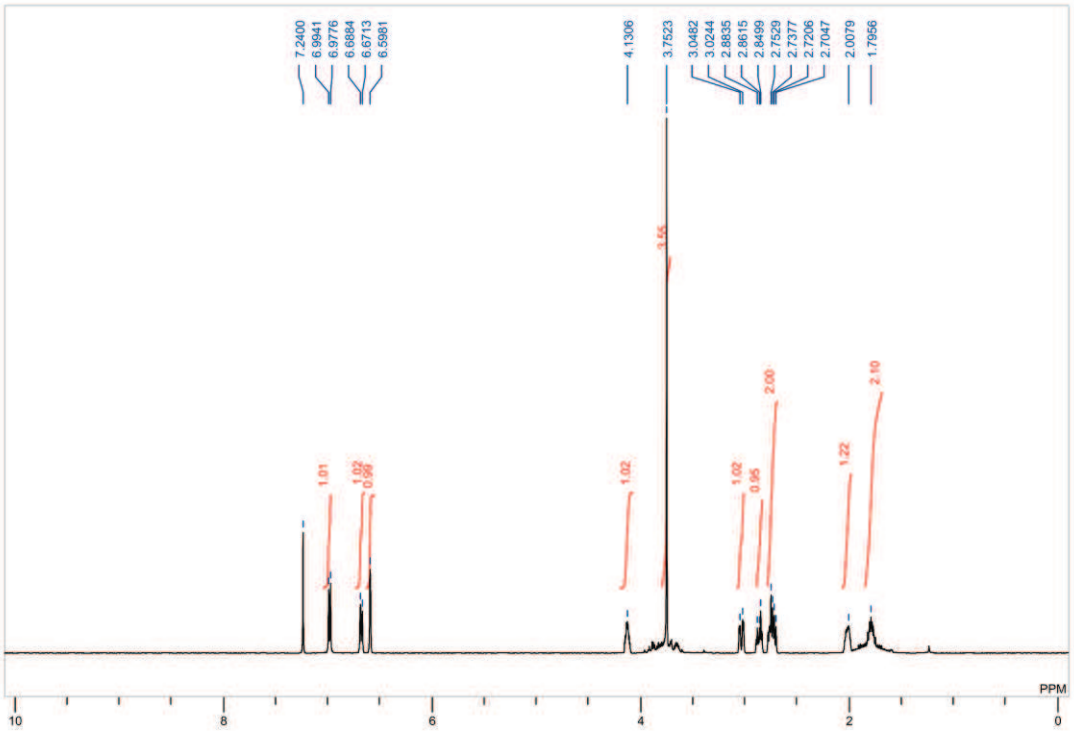


Figure A32. ^{1}H NMR spectrum of the (*S*)-**2c** prepared by W110G TeSADH.

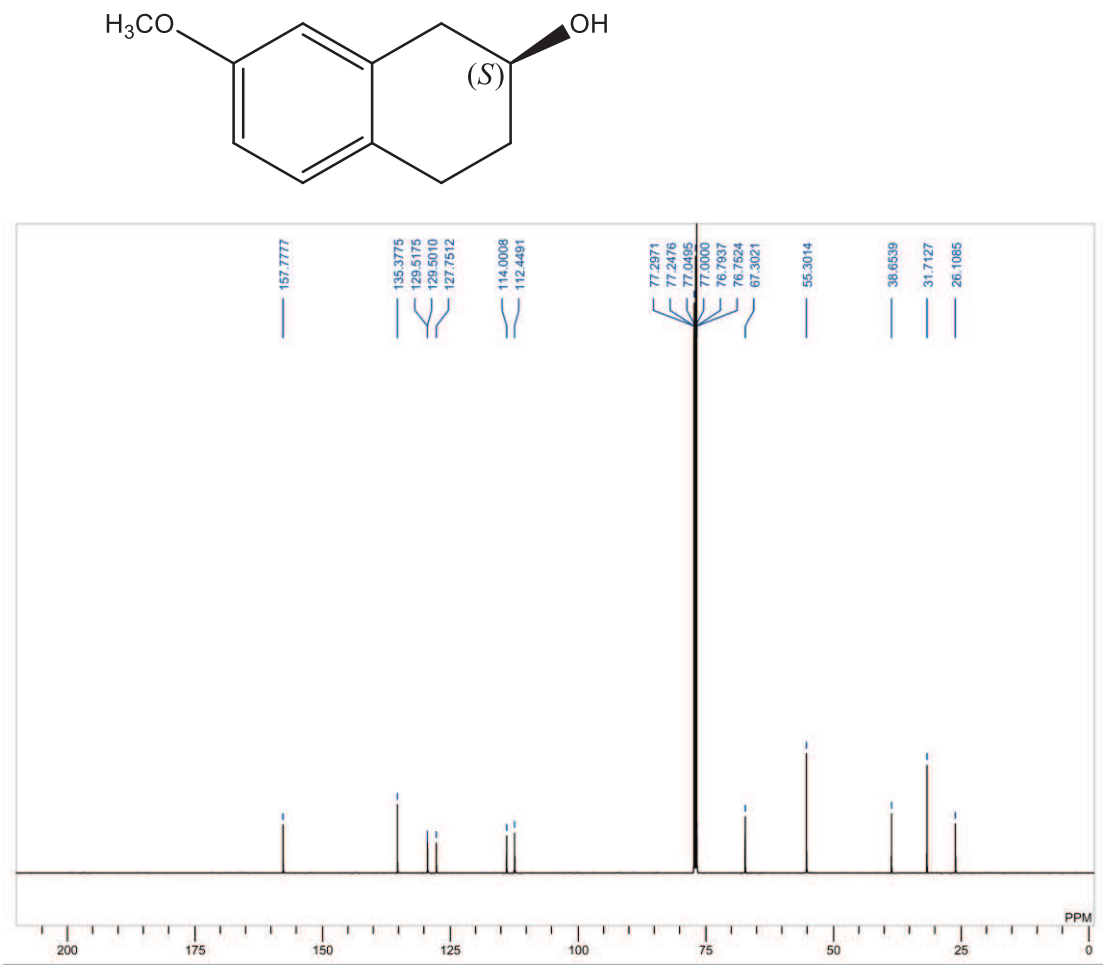


Figure A33. ^{13}C NMR spectrum of the (*S*)-**2c** prepared by W110G TeSADH.

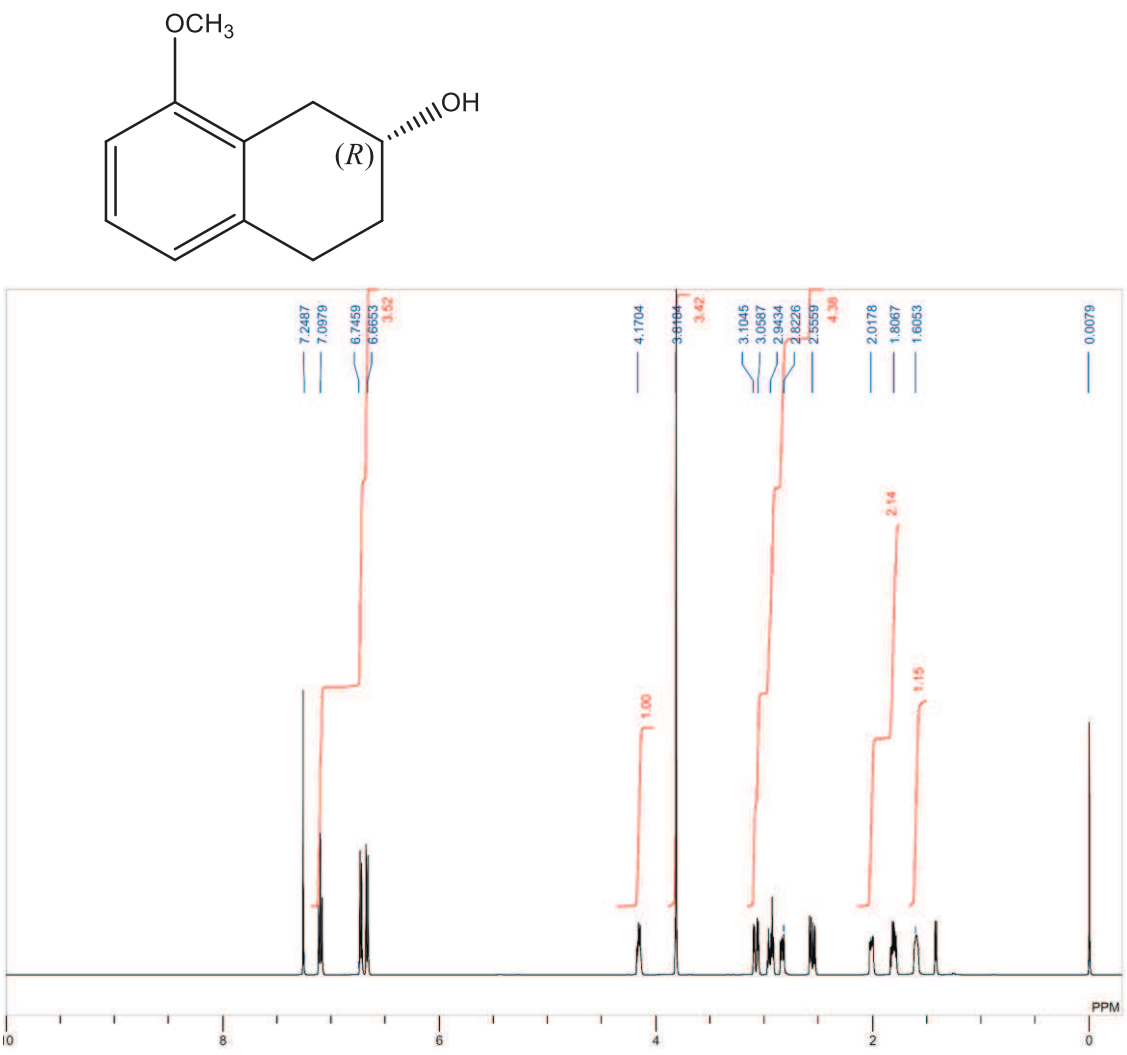


Figure A34. ^{1}H NMR spectrum of the (*R*)-**2d** prepared by W110G TeSADH.

Figure A35. ^{13}C NMR spectrum of the (*R*)-**2d** prepared by W110G TeSADH.

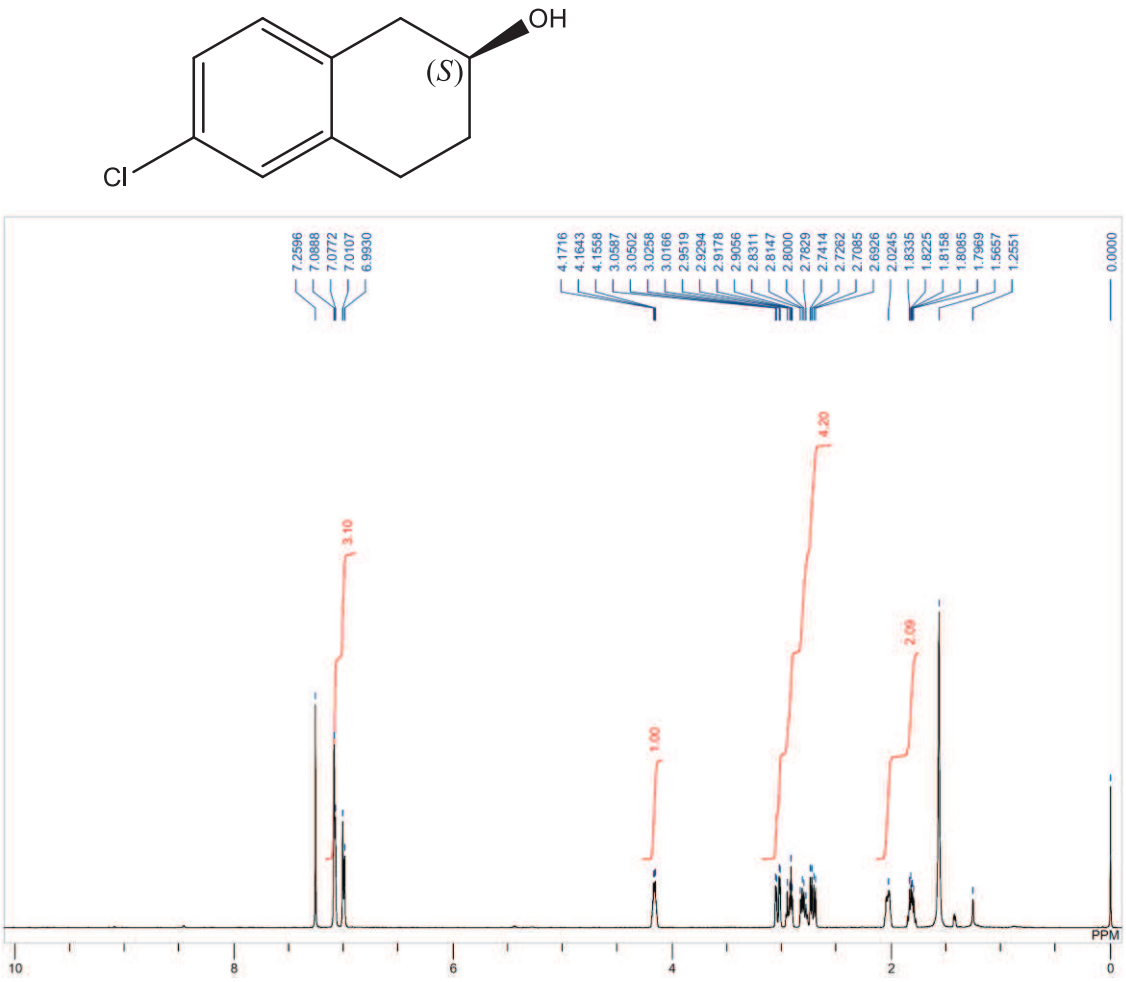


Figure 36. ^{1}H NMR spectrum of the (*S*)-**2e** prepared by W110G TeSADH.

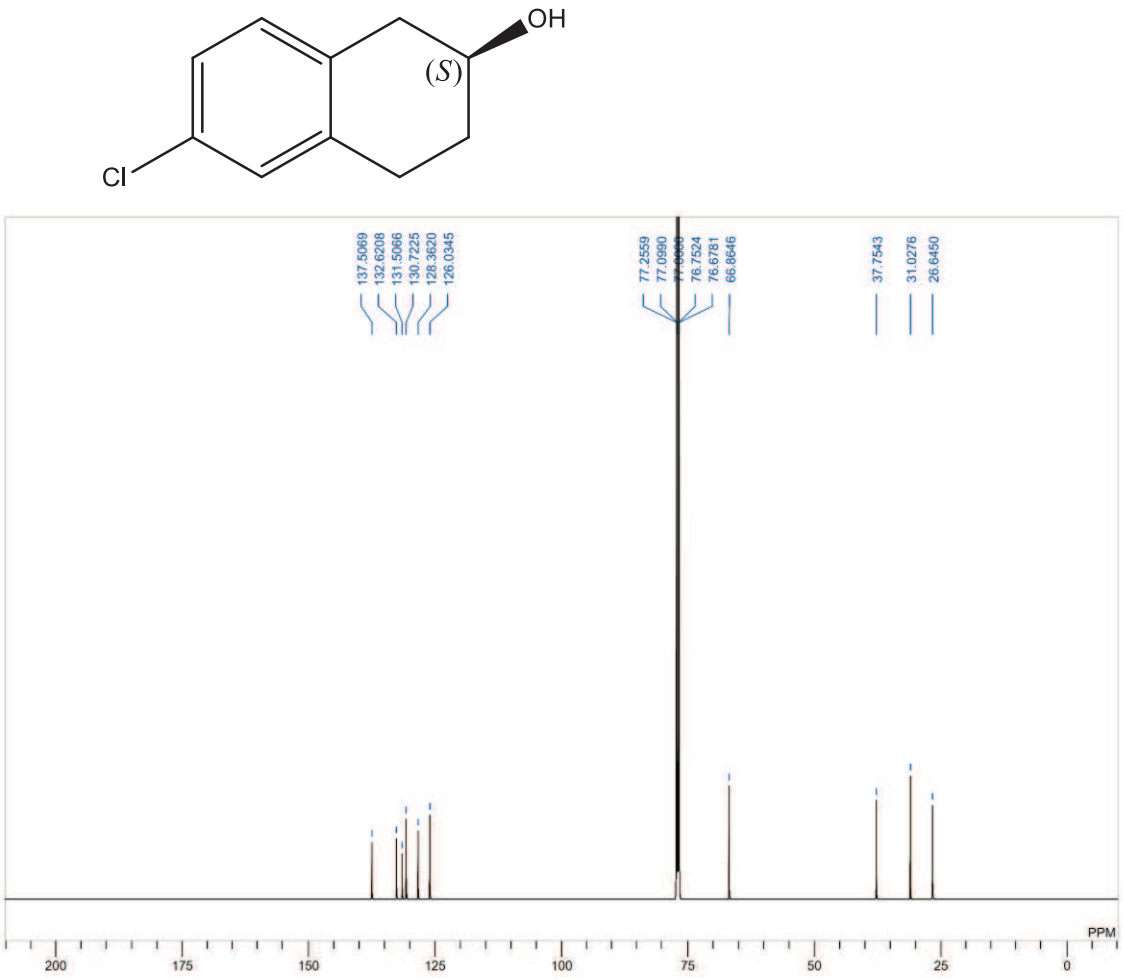
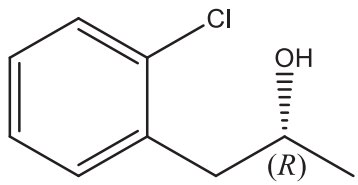


Figure A37. ^{13}C NMR spectrum of the (*S*)-**2e** prepared by W110G TeSADH.

Figure A38. ^{1}H NMR spectrum of the (*R*)-**2l** prepared by W110A/I86A TeSADH.



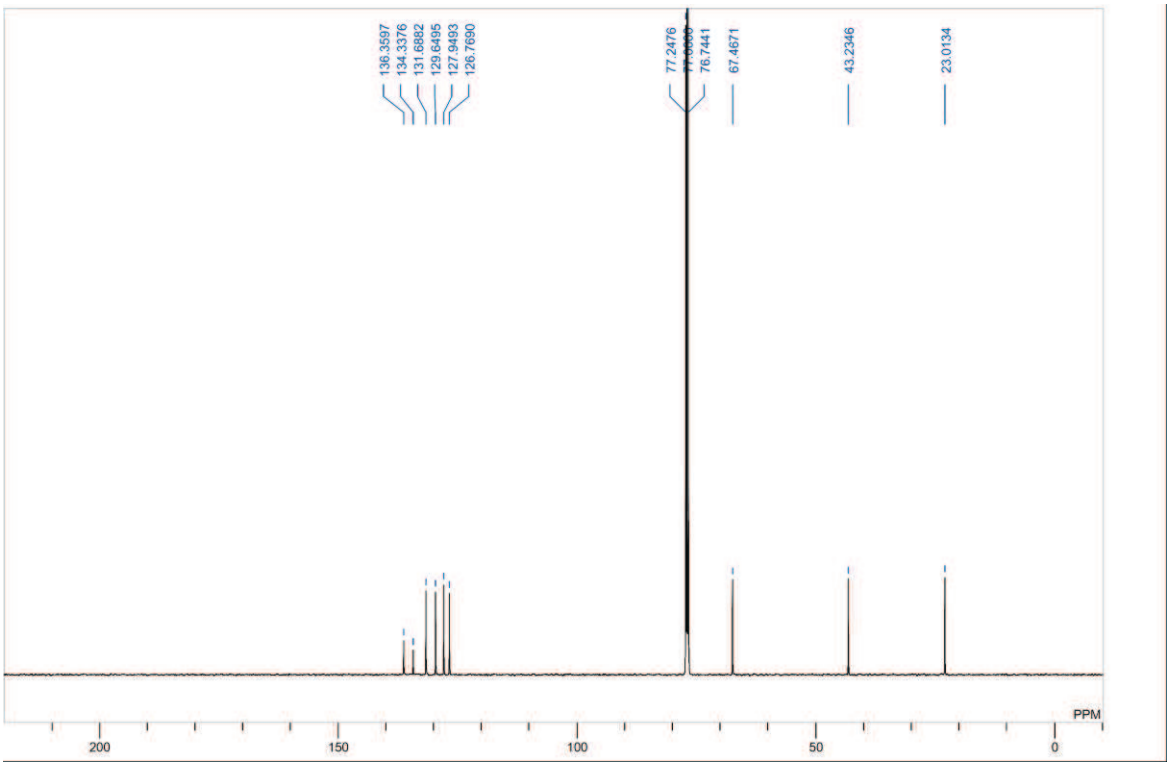
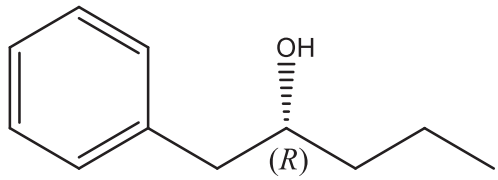


Figure A39. ^{13}C NMR spectrum of the (*R*)-**2l** prepared by W110A/I86A TeSADH.



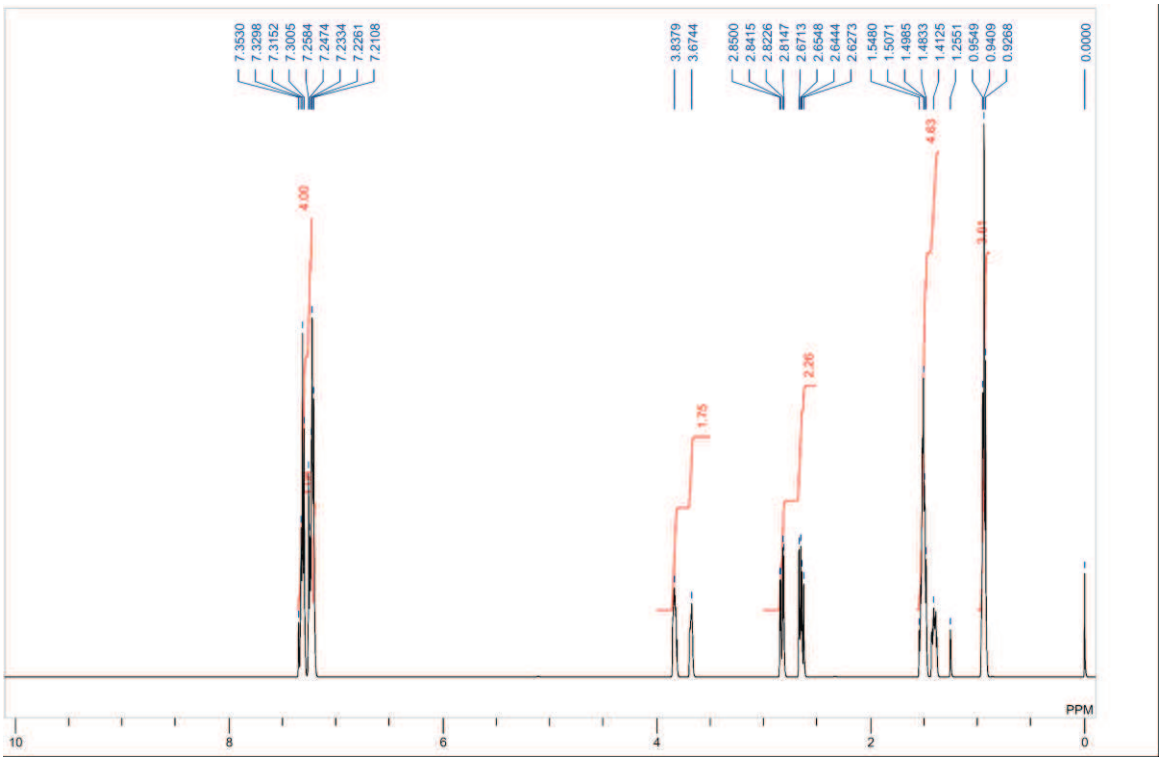


Figure A40. ^{1}H NMR spectrum of the (*R*) **-2o** prepared by W110A/I86A TeSADH.

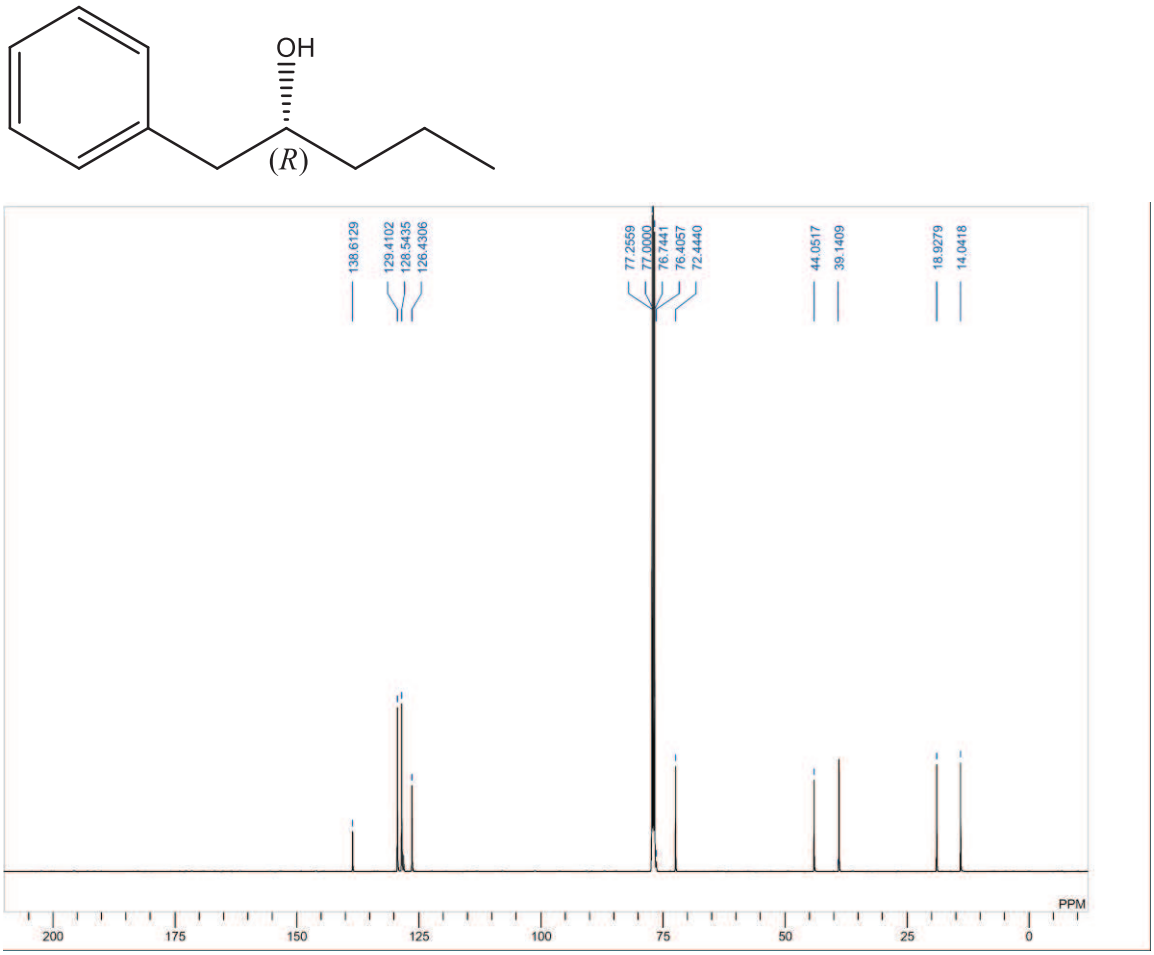


Figure A41.[13]C NMR spectrum of the *(R)* -**2o** prepared by W110A/I86A TeSADH.

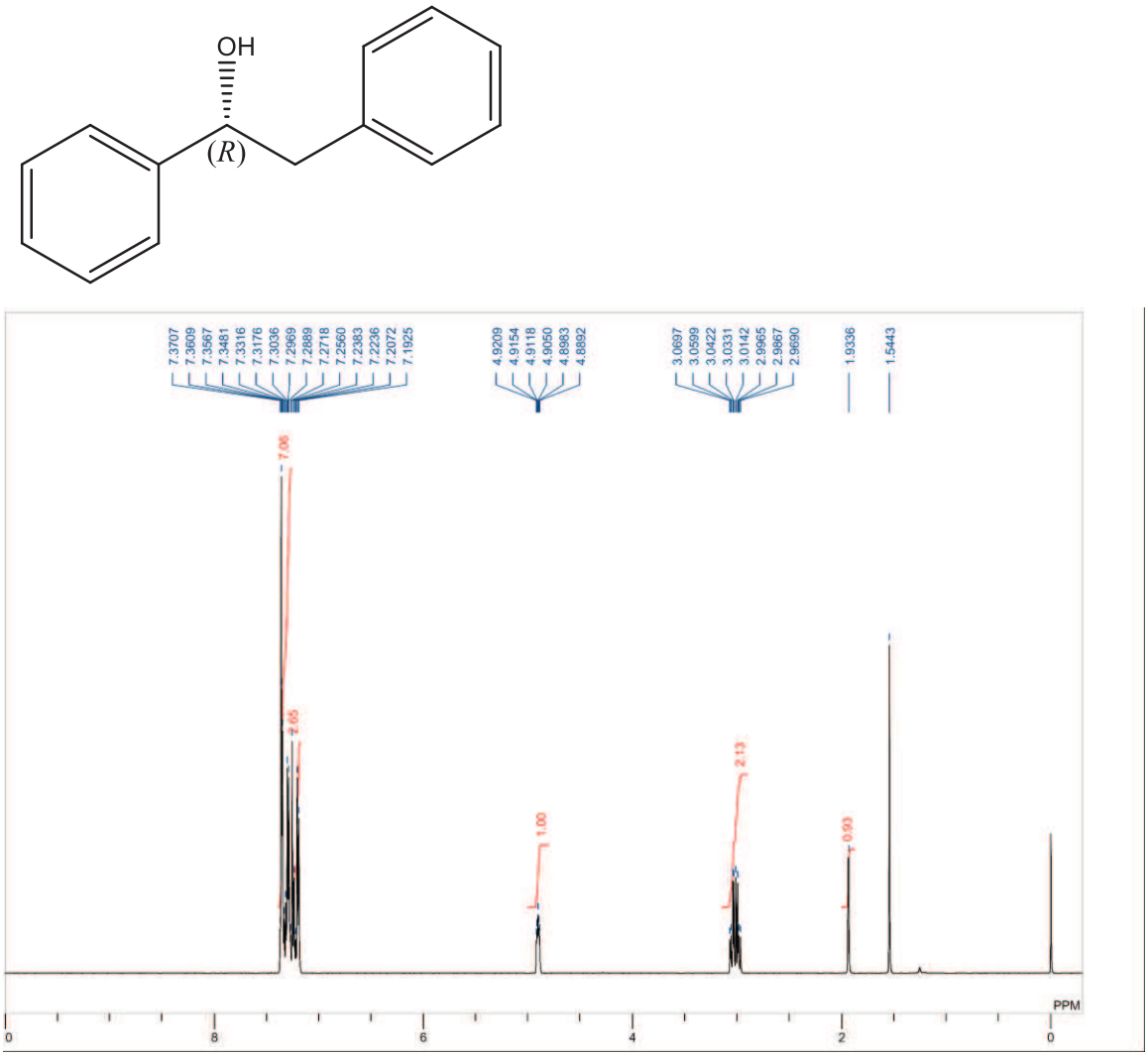


Figure A42. ^{1}H NMR spectrum of the (*R*) – **2q** prepared by W110A/I86A TeSADH.

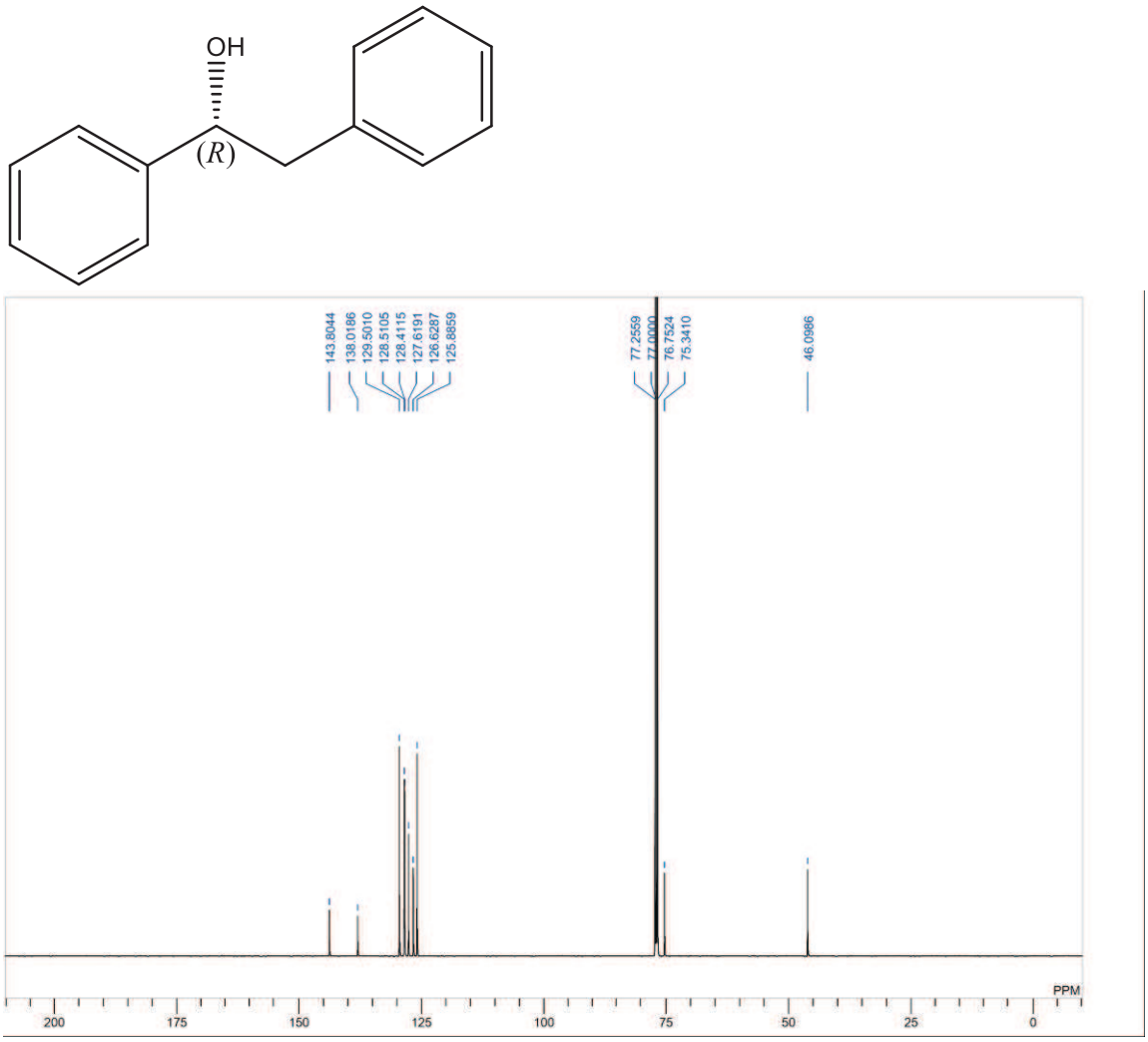


Figure A43. ^{13}C NMR spectrum of the (*R*) **-2q**prepared by W110A/I86A TeSADH.

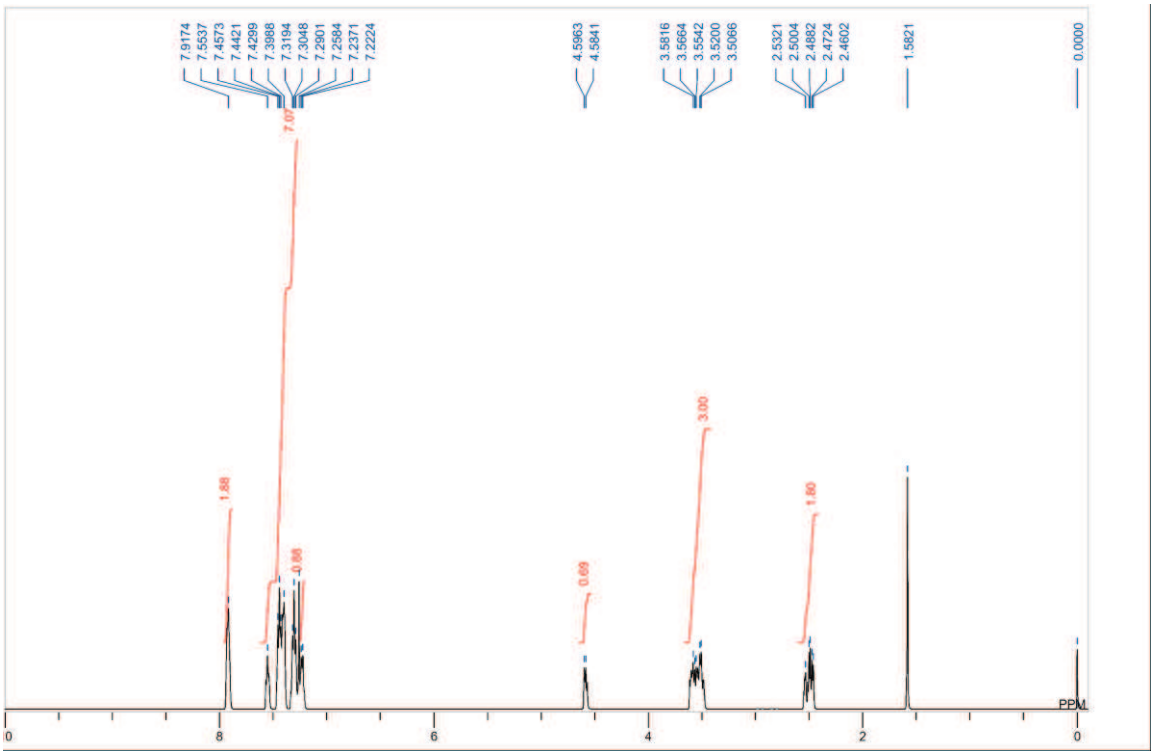


Figure A44. [1]H NMR spectrum of the **2r** prepared by W110A/I86A TeSADH.

Figure A45. ^{13}C NMR spectrum of the **2r** prepared by W110A/I86A TeSADH.

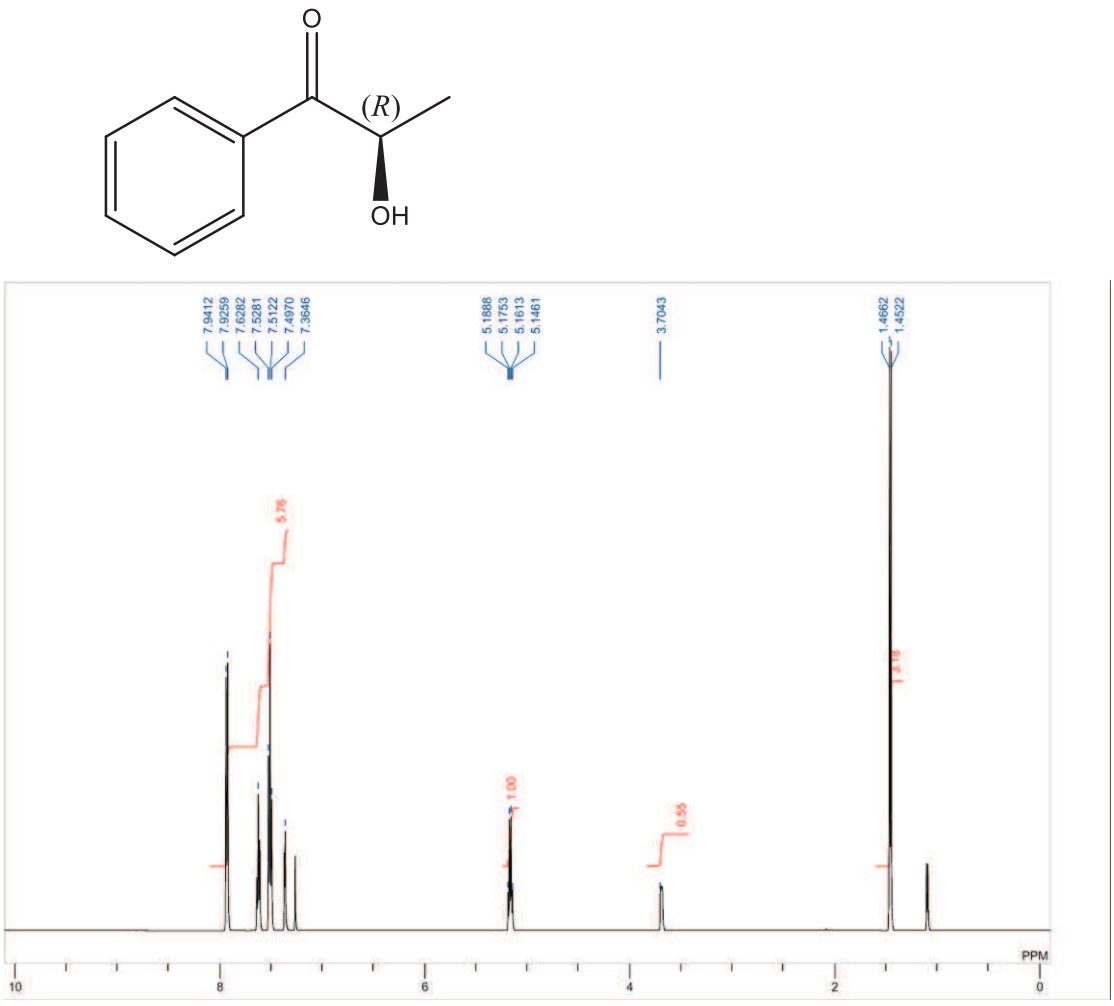
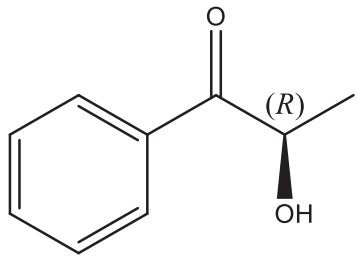


Figure A46. ^{1}H NMR spectrum of the (*R*) **-2s** prepared by W110A/I86A TeSADH.



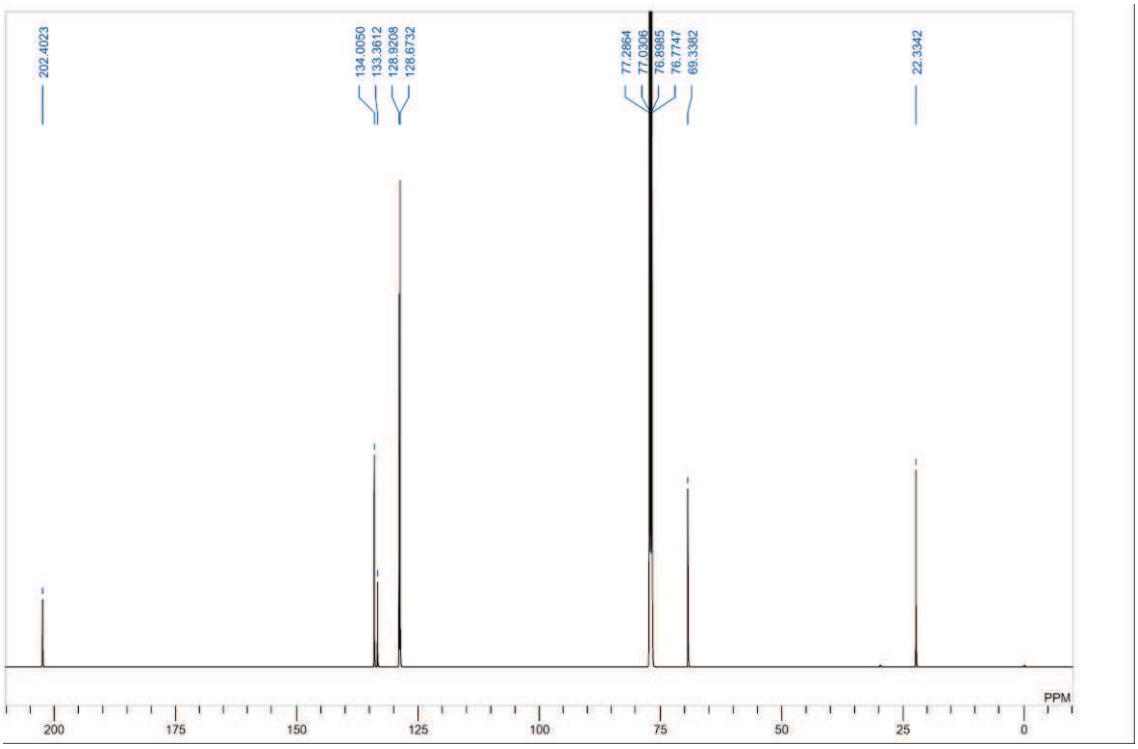


Figure A47. ^{13}C NMR spectrum of the (*R*) – **2s** prepared by W110A/I86A TeSADH.

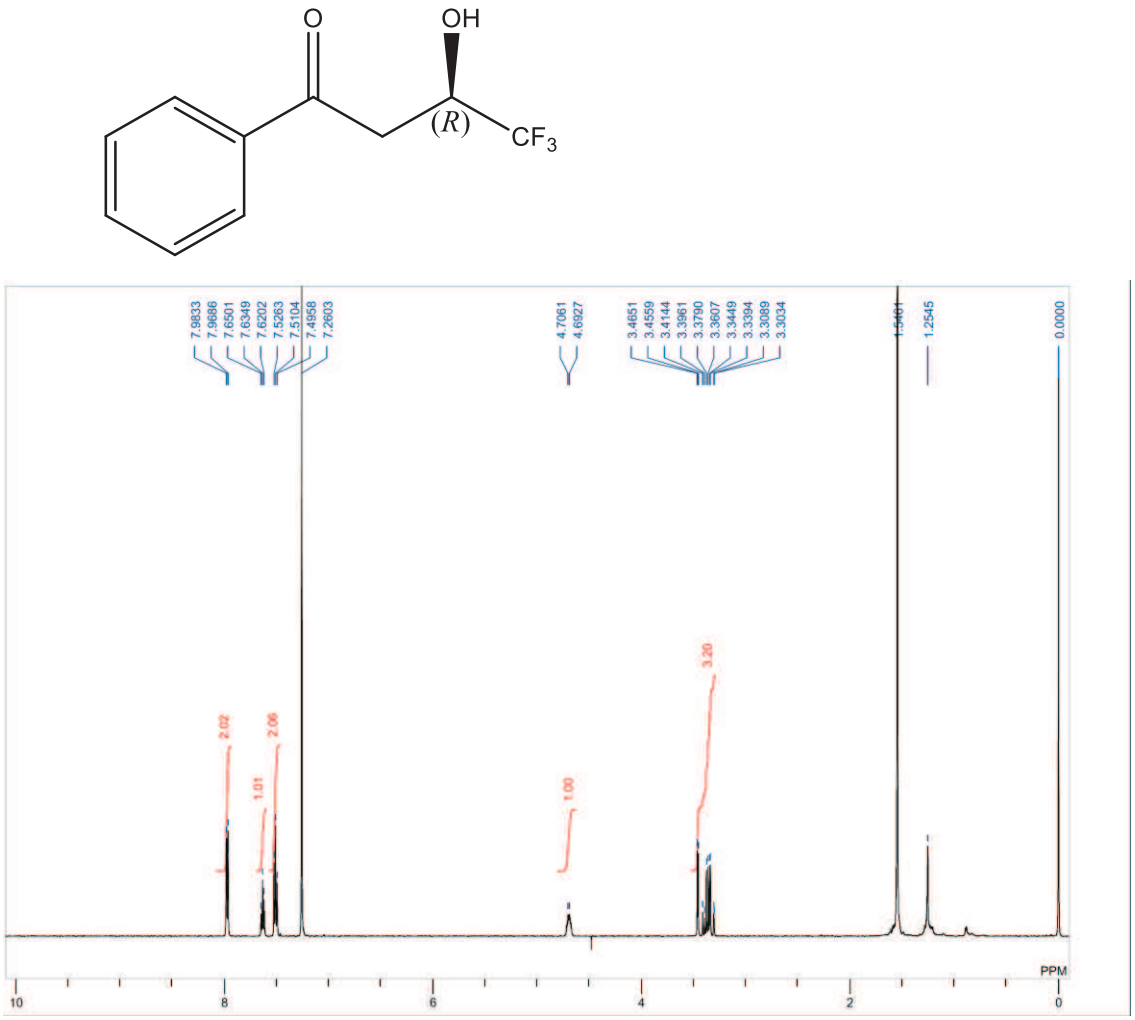
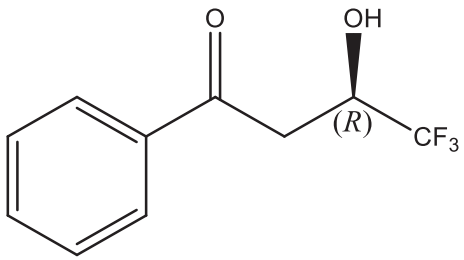


Figure A48. ^{1}H NMR spectrum of the (*R*) – **2v** prepared by W110V TeSADH.



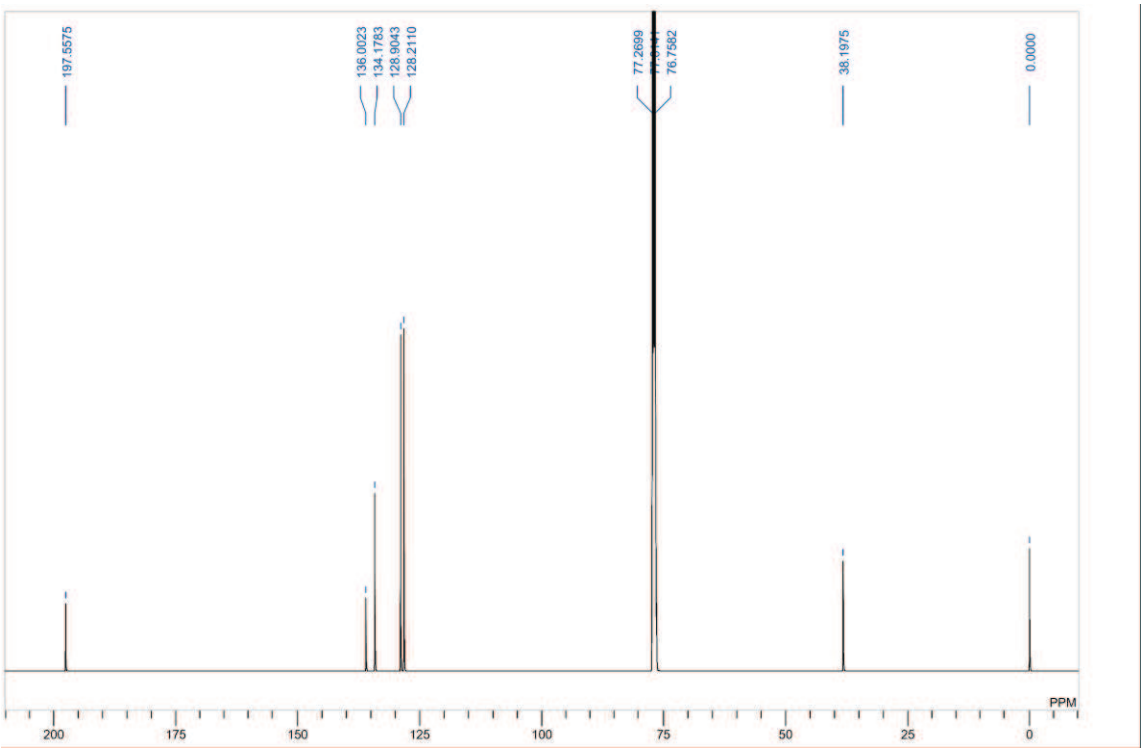


Figure A49. ^{13}C NMR spectrum of the (*R*) – **2v** prepared by W110V TeSADH.

4. FT-IR spectra

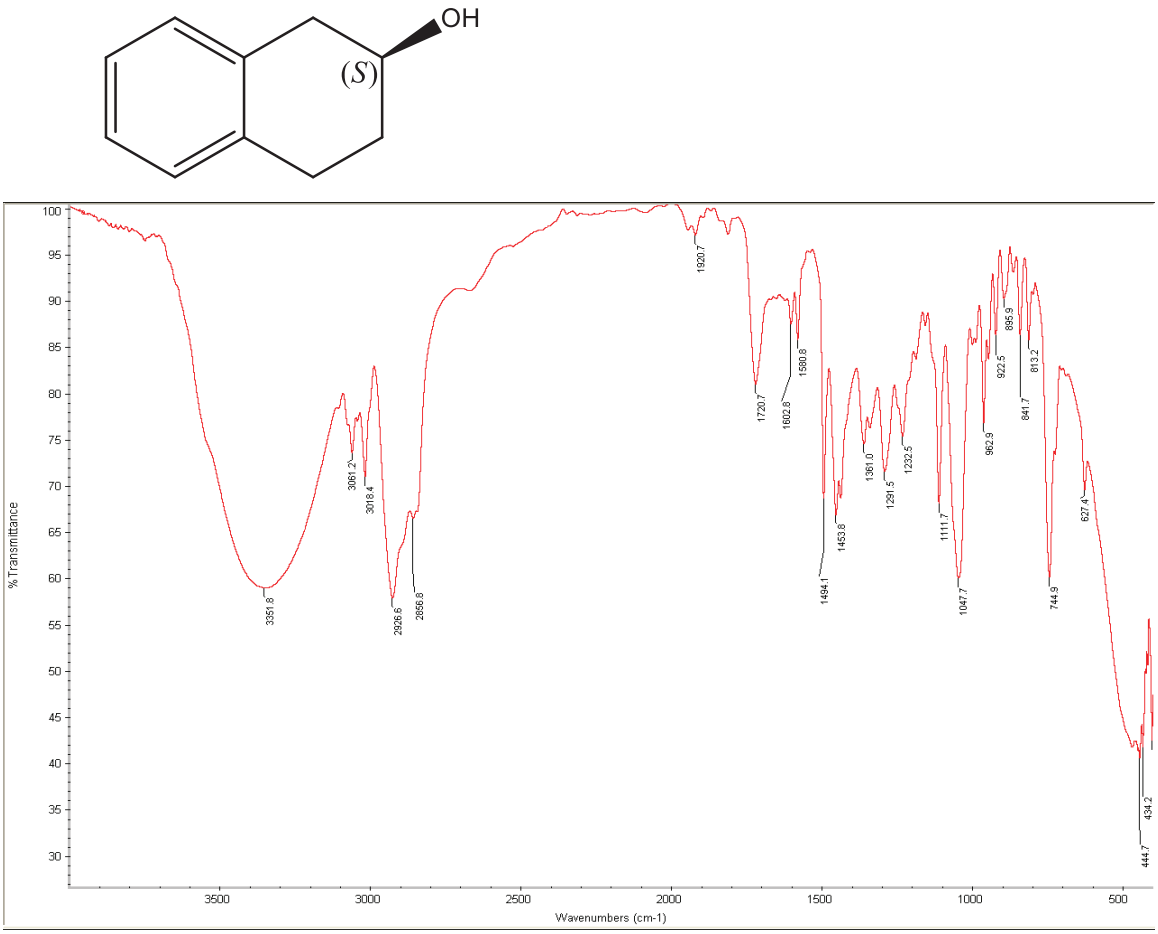


Figure A50. FT-IR spectrum of the (*S*)-**2a** prepared by W110G TeSADH.

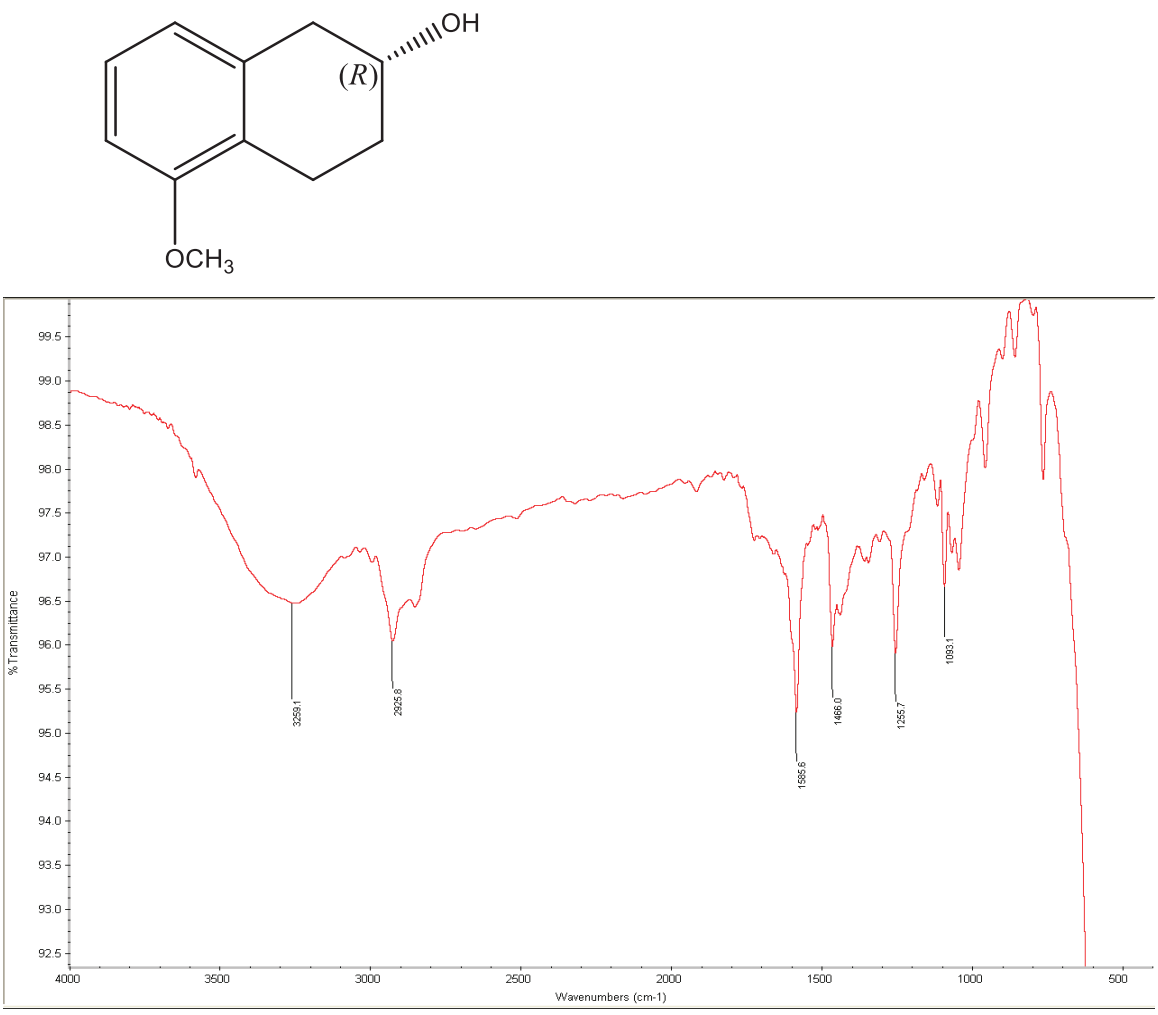


Figure A52. FT-IR spectrum of the (*R*)-**2b** prepared by W110G TeSADH.

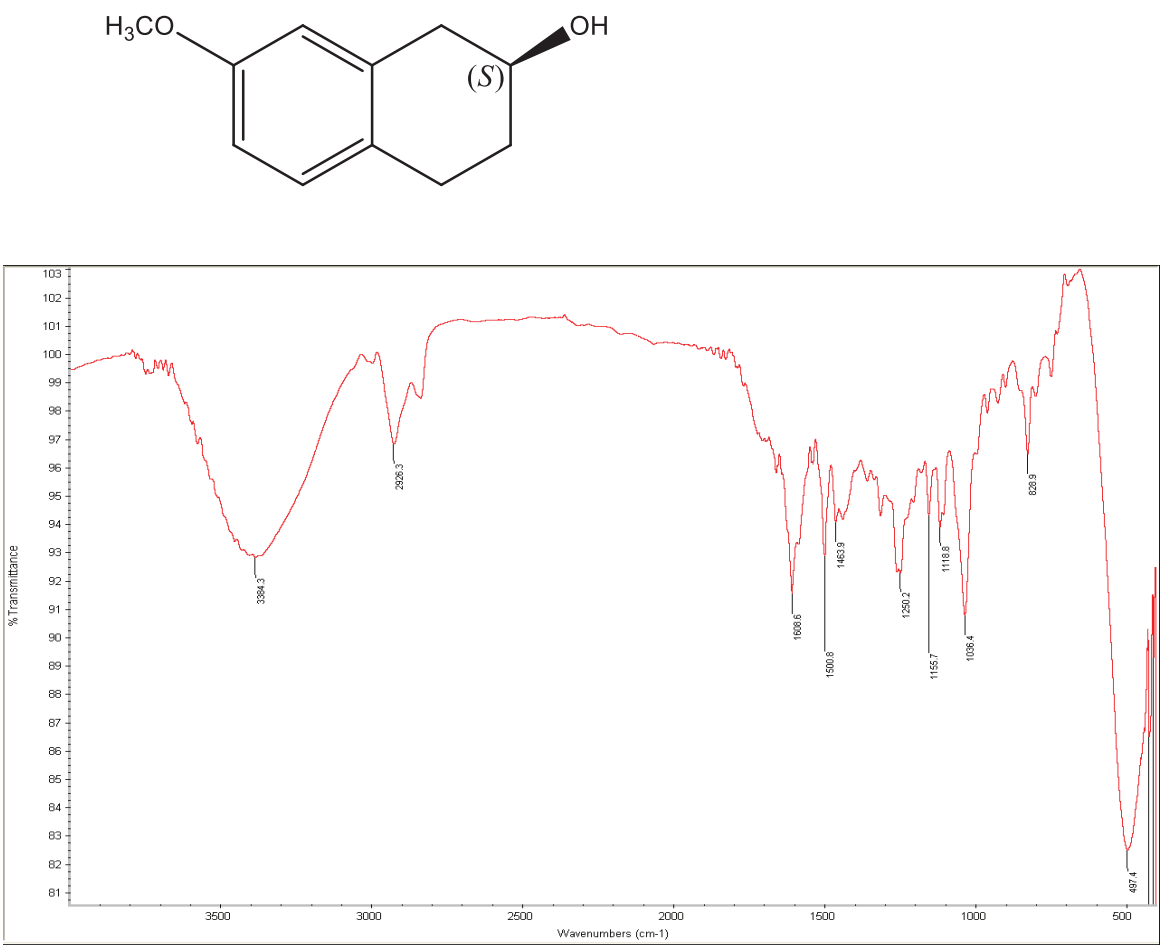


Figure A53. FT-IR spectrum of the (*S*)-**2c** prepared by W110G TeSADH.

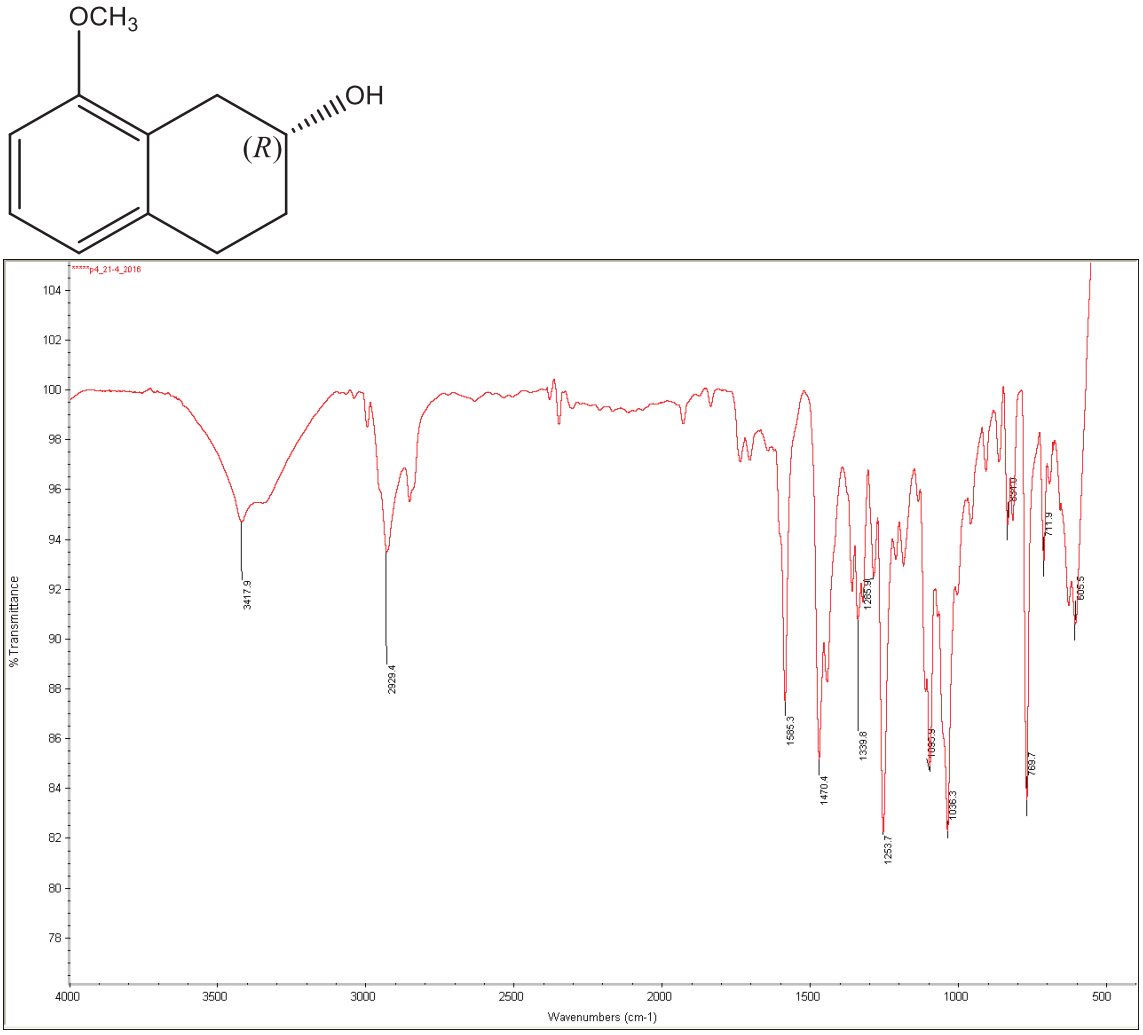


Figure A54. FT-IR spectrum of the (*R*)-**2d** prepared by W110G TeSADH.

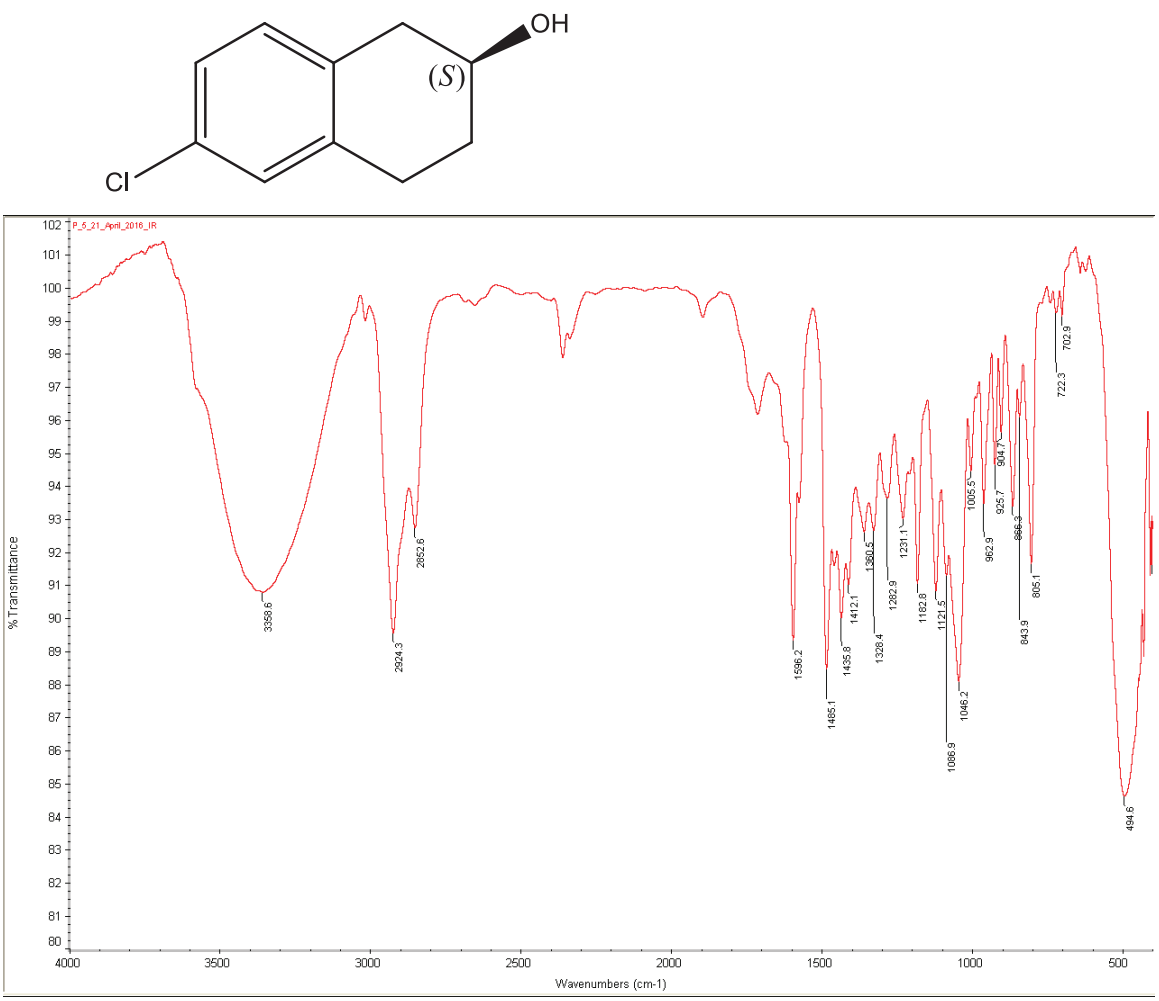
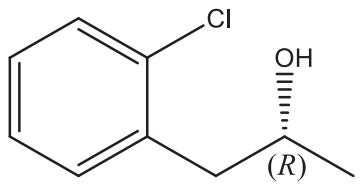


Figure A55. FT-IR spectrum of the (*S*)-**2e** prepared by W110G TeSADH.



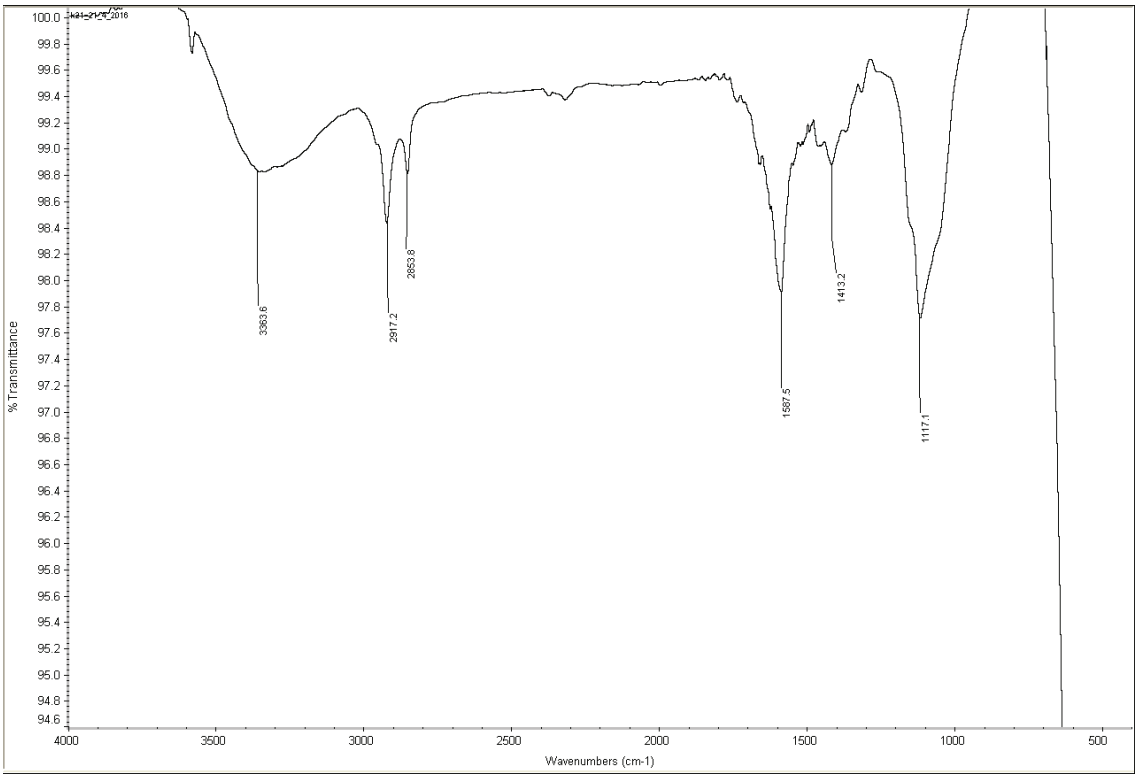
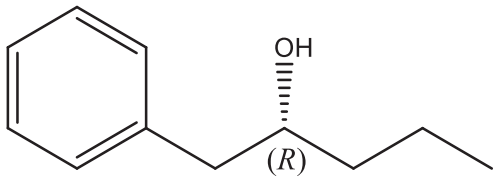


Figure A56. FT-IR spectrum of the (*R*) **–2l** prepared by W110A/I86A TeSADH.



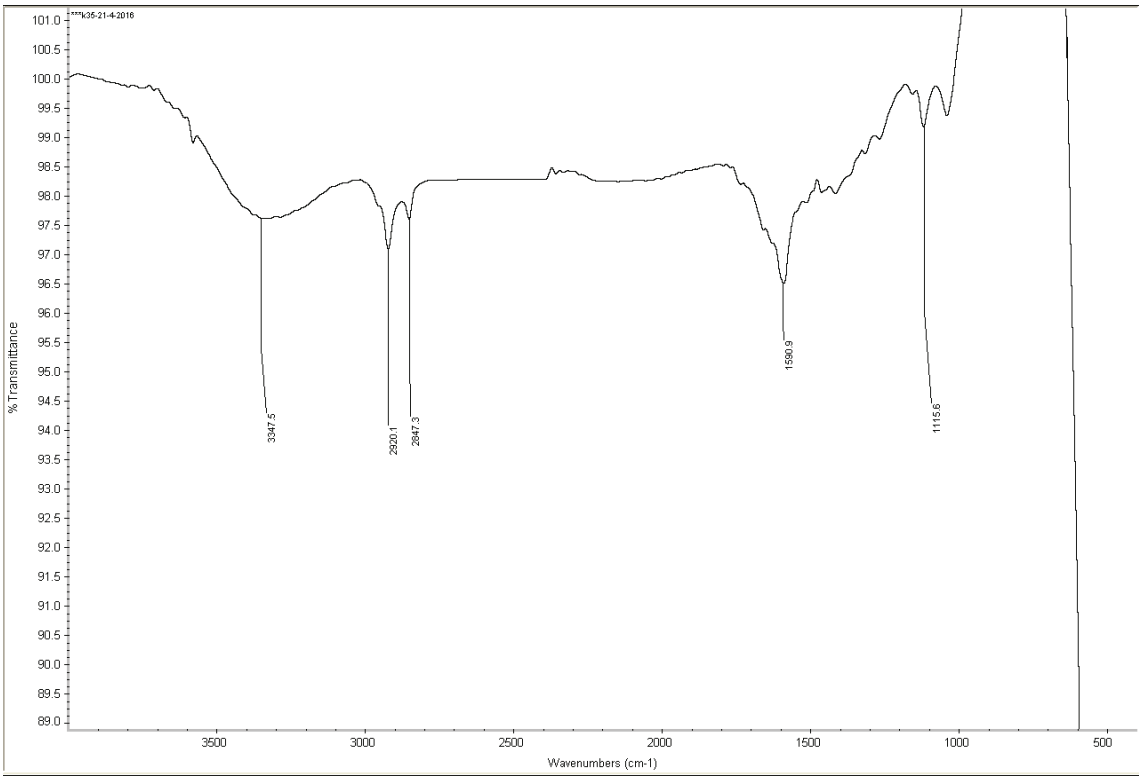


Figure A57. FT-IR spectrum of the (*R*)-**2o** prepared by W110A/I86A TeSADH.

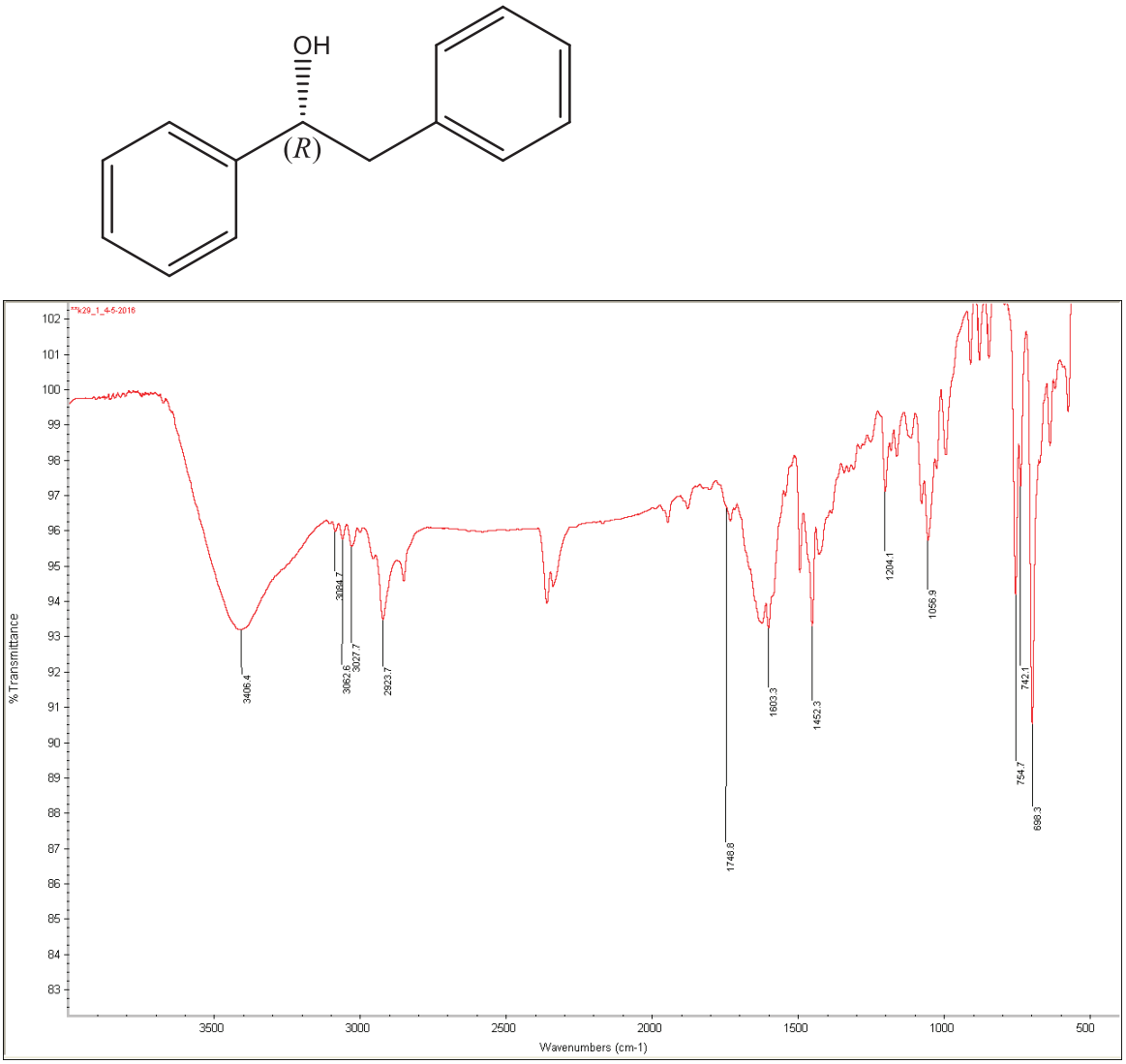


Figure A58. FT-IR spectrum of the (*R*) – **2q** prepared by W110A/I86A TeSADH.

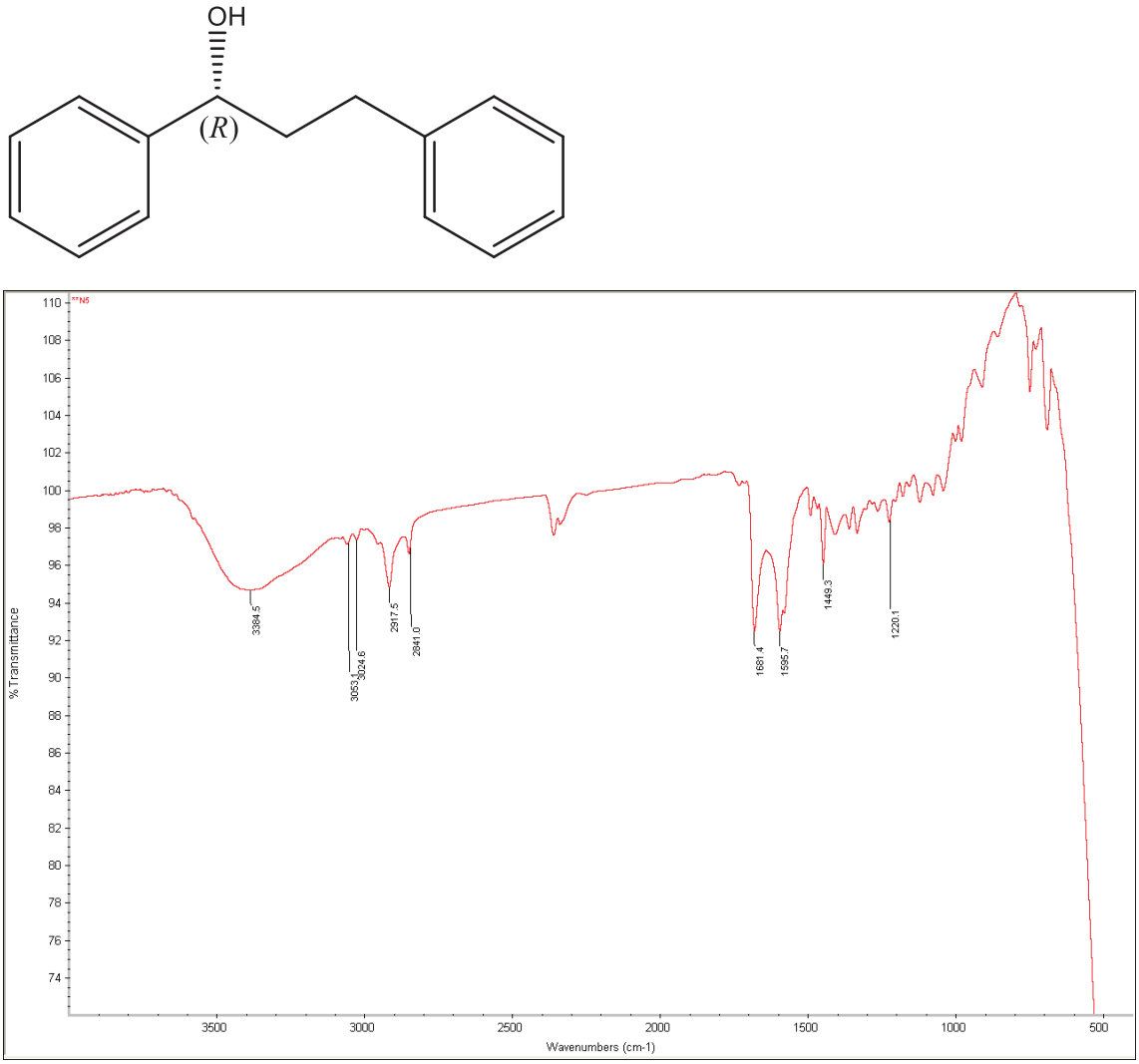


Figure A59. FT-IR spectrum of the **2r** prepared by W110A/I86A TeSADH.

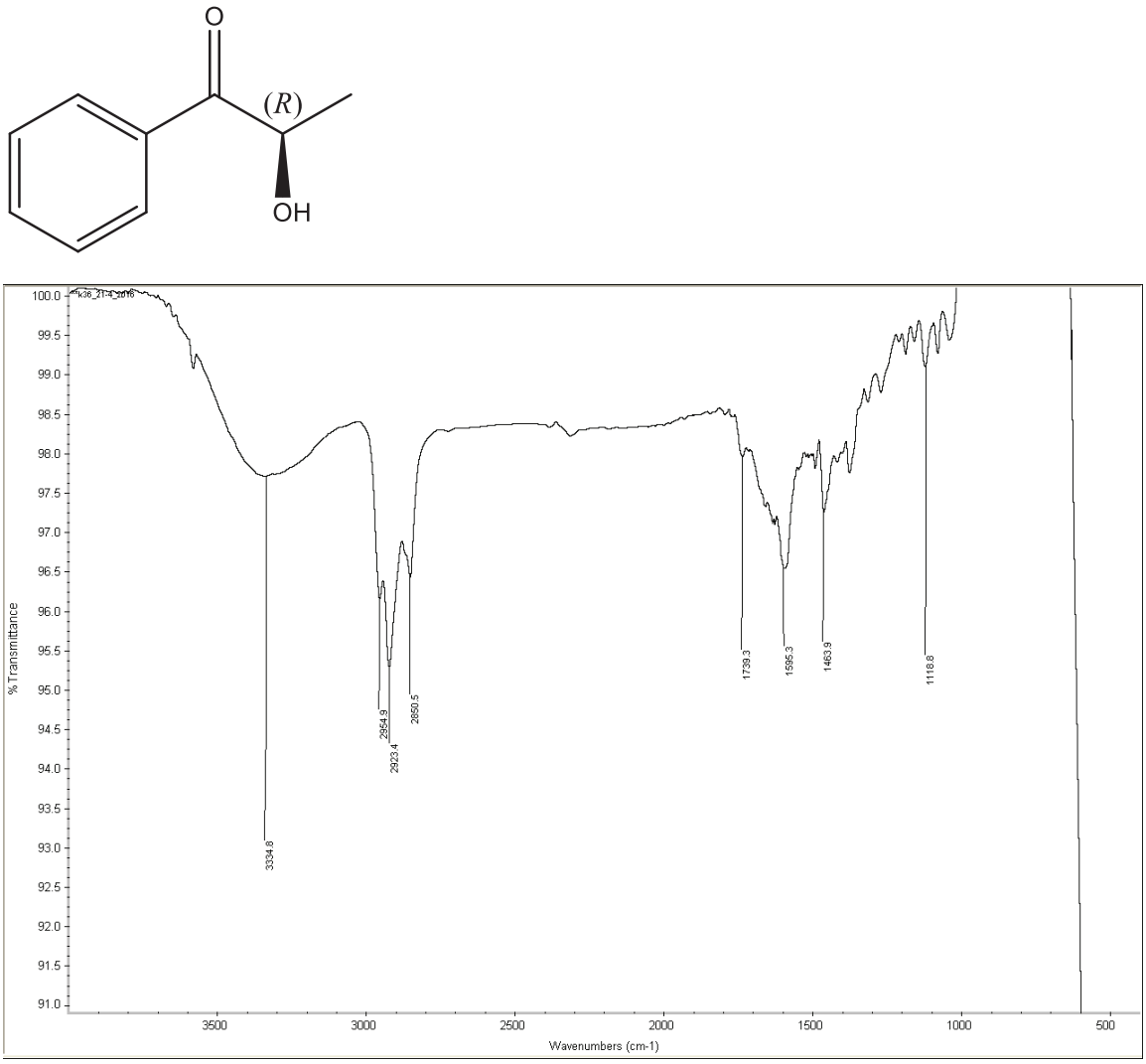


Figure A60. FT-IR spectrum of the (*R*) **–2s** prepared by W110A/I86A TeSADH.

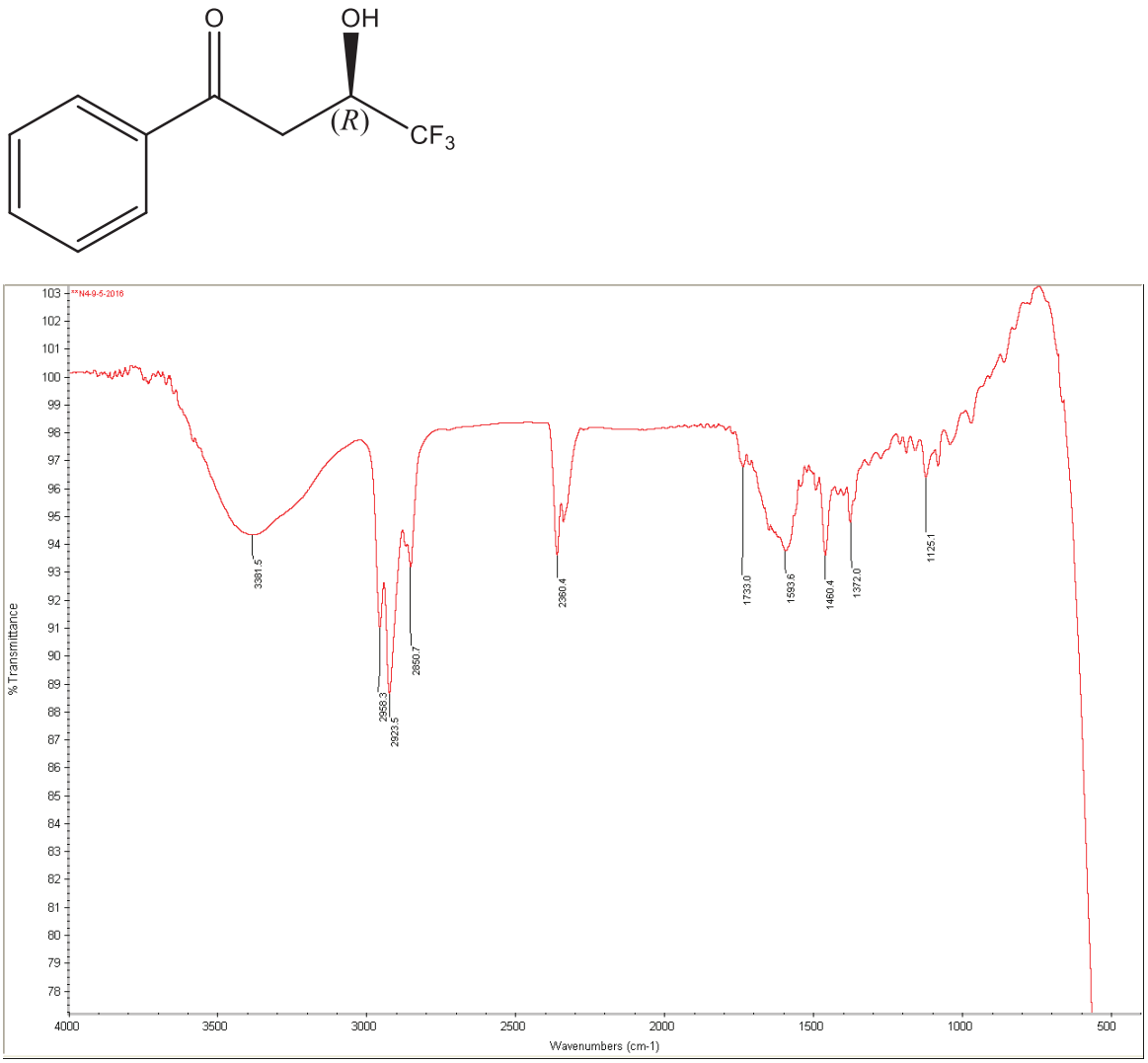


Figure A61. FT-IR spectrum of the (*R*) **-2v** prepared by W110V TeSADH.

Vitae

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